NUTRITIONAL EFFECTS ON THE GASTRIC MUCOSA OF THE RAT

I. LESIONS OF THE ANTRUM

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FIVE FIGURES

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More than fifty reports can be cited in the literature of the last two decades which deal with gastric lesions of nutritional origin. Fridericia et al. ('40) and Sharpless ('40; '43) have reviewed the literature from different points of view. Attempts to establish specific deficiencies as responsible causes have not led to very acceptable conclusions. An element of complexity is introduced by the fact that three types of lesions have been identified (Berg, '42), each characteristic of one of the three types of epithelial coats which make up the gastric mucosa of the rat (figs. 1, 2 and 4) of this and figs. 1–3 of the succeeding paper). It appears that less progress has been made in clarifying the relations of individual factors to gastric epithelium than has occurred in the more recent field of study which deals with skin epithelium and the factors necessary for its normal maintenance.

Orienting experiments were begun several years ago with repetitions of the experiments of other investigators. While our first observations were based on frankly multiple deficiency diets (e.g., diet 872 of table 2) on which all three types of lesions can be produced in the same rat, our aim was to identify individual deficiencies with the abnormal behavior of the epithelium. We have published two brief reports on antral lesions and calcium deficiency (Zucker and Berg, '43; '44). This and the following paper deal with more detailed observations on all three regions of the stomach.

TECHNIQUE

To prepare a stomach for observation the esophagus is cut at its entrance to the stomach and about 1 cm. of duodenum is left attached. The stomach is then cut open along the greater curvature and stretched out with pins on cardboard prior to fixation in 4% formaldehyde solu-

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tion (a 1 to 10 dilution of "formalin"). This places the region of the lesser curvature (called antrum or prepyloric region) in the center with the two parts of the cut fundus (also called body or fornix) on the sides and the rumen (forestomach) above. The esophageal opening is in the center of such a stretched specimen. Figure 1 shows a normal rat's stomach photographed against a white background to show the

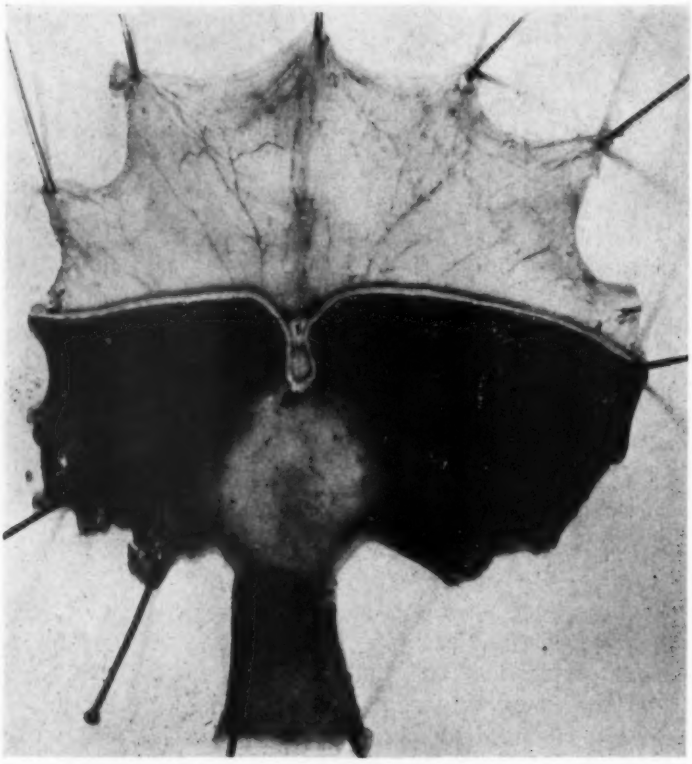

Fig. 1 Stomach of a normal rat opened along the greater curvature and stretched out flat on cardboard. This stomach was photographed against a white background and in the fresh state thus retaining the color in order to differentiate the various regions. All the other specimens in this and the succeeding paper were fixed in formalin and have lost the natural color of the fundus. They were photographed against a black background so that the lesions would show to better advantage. The transparent area above the dividing ridge is the rumen (forestomach). Below the ridge is the glandular part of the stomach. Separated from this by the pylorus is the duodenum. The glandular area is subdivided into the fundus (dark, thicker) and the antrum (translucent, thinner). The esophagus enters near the juncture of the three regions. In the rat the antrum secretes no acid whatsoever — only mucus. The glands of the fundus contain the acid and pepsin secreting cells. This region is also the most vascular.

transparent nonsecretory rumen above the dividing ridge which separates it from the glandular portion consisting of the acid secreting fundus and the non-acid mucus secreting antrum. The latter portion of the stomach has a smooth translucent appearance. In the normal stomach the only interruption of the smooth surface consists of lymph nodules which can be distinguished from lesions in microscopic section and which when counted in groups of animals average one or less per rat.

The appearance of the typical antral lesions of calcium deficiency is shown in figure 2. The circumscribed raised areas are due to hyperplasia of the glandular epithelium. The center usually shows a pin-

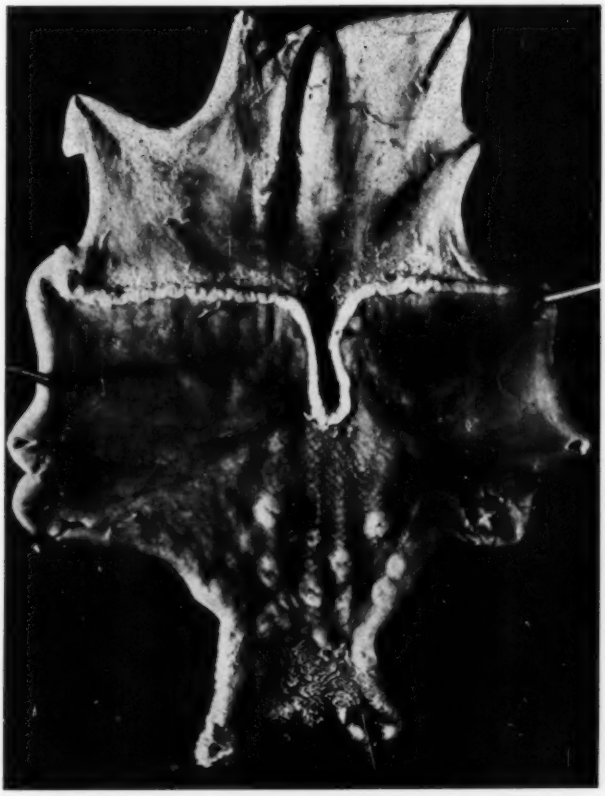

Fig. 2 Antral lesions (4-week Ca deficiency). Rumen and fundus are normal. Lesions in the antrum are sharply circumscribed raised areas with central pits or grooves which may show either a white or red central dot or may be clear. The location of most of the lesions near the pylorus, as in this figure, is rather typical although at times the whole antral area may be covered.

point hemorrhage or a white plug consisting of fibrin and leucocytes with hemoglobin no longer present. The variation in size of the individual lesions in a given stomach is not always as large as shown in figure 2. Without microscopic examination of sections a lymph nodule cannot always be distinguished from a hyperplastic lesion. In the tables the severity of the condition is expressed by the incidence figure (i.e., fraction of animals showing abnormal antrum) and a mean count of lesions per rat.[1]

TABLE 1

Production of lesions by calcium deficiency.

SERIES [1]		ADDED		WEIGHT [2] IN GM. AT		NO. OF RATS	ANTRUM	
		Ca	P	28 days	56 days		Count	Incidence [3]
1	Control	0.6	0.4	49	105	124	0.5	4%
	Experimental	0.0	0.4	53	77	134	11.9	88%
2	Control	0.6	0.4	65	174	94	0.2	2%
	Experimental	0.0	0.4	65	101	141	13.6	85%

[1] Series 1 has 20% protein, series 2 has 27%. Series 2 has more B complex than series 1. For composition of diets see table 2; diets 904, 905 and 1009 are typical members of series 1 and diets 1015–1017 and 1029 are typical members of series 2.

[2] Sexes are equally distributed in each group. There is always a large sex difference in growth in the control diets of series 2, less difference in the control diets of series 1 (due to protein content which is suboptimal for males, Zucker and Zucker, '43; '44) and little sex difference on the Ca deficient diets. The rats in series 2 were larger throughout. On the same stock diet within the same rat strain the breeders had been selected from large sized parents for several generations.

[3] See footnote 1 below.

EFFECT OF CALCIUM DEFICIENCY

Table 1 presents two series of experiments, comprising a total of 493 rats. In each series the salt mixture of the control diets contributes 0.6% Ca, while in the experimental diets calcium addition was omitted. Nearly 90% of the experimental animals showed a definitely abnormal antrum, while the control diets resulted in a normal gastric mucosa

[1] In the incidence figure we consider an antrum not normal when more than one raised area is seen. This introduces less error than the inclusion of normal lymph nodules. The mean count includes the lymph nodules and is of interest only if well removed from unity. Statistical comparisons of groups can be based on either kind of observation, using "t" for mean count and chi square for incidence. The mean count appears to be by far the more precise measure. We give the incidence also because it has been the more customary measure and is the only one which applies in studies of rumen and fundus.

which did not differ from that in the 198 animals of corresponding age that were fed the stock diet. Included among the controls of the second series are forty-eight rats on diets in which the B factors were supplied in the form of liberal allowances of thiamine, riboflavin, pyridoxine, pantothenic acid and in some cases choline. As stated elsewhere on the basis of a smaller series (Zucker and Berg, '43), the experimental animals of series 2 showed lesions exclusively in the antrum, while those of series 1 also had defects in other parts of the gastric mucosa. The nature and incidence of the latter are discussed elsewhere (Zucker, Berg and Zucker, '45).

TABLE 2

Composition of diets.[1]

DIET	PROTEIN	DIETARY Ca	DIETARY P	B COMPLEX SUPPLIED	CARBOHYDRATE
		%	%		
872	12% Labco casein	0.80	0.34	.....	starch
896	20% Labco casein	0.60	0.41	.....	sucrose
904	20% Egg albumen	0.61	0.45	5% Vitab	sucrose
905	20% Egg albumen	0.010	0.45	5% Vitab	sucrose
909	20% Egg albumen	0.012	0.48	10% Vitab, 1.5 mg. B_2	sucrose
913	20% Egg albumen	0.010	0.044	5% Vitab	sucrose
1009	20% Fibrin	0.044	0.49	10% Vitab, 1.5 mg. B_2	sucrose
1010	20% Fibrin	0.64	0.43	.....	sucrose
1015	27% Labco casein	0.60	0.41	5% Vitab, 0.1 mg. B_2, 0.5% yeast extract	cerelose
1016	27% Labco casein	0.0037	0.41	5% Vitab, 0.1 mg. B_2, 0.5% yeast extract	cerelose
1017	27% Labco casein	0.0078	0.46	5% Vitab, 0.1 mg. B_2, 2.0% yeast extract	cerelose
1019	27% Labco casein	0.60	0.40	.....	starch
1029	27% Labco casein	0.017	0.41	5% yeast extract	cerelose
1035	27% Labco casein	0.24	1.24	5% Vitab, 0.25 mg. B_2, 0.75 mg. Ca pantothenate	cerelose

[1] All diets in this and the following paper have a total of 2% added fat, in the form of refined cottonseed oil containing 750 units vitamin A activity as carotene. Diets 872, 896, 1010 and 1019 contain 0.3% cod liver oil (replacing an equal weight of cottonseed oil). Diets 872 and 896 have 1% NaCl. All other diets contain 1.2% modified (Ca, P free) Wesson salt mixture. Ca is added as $CaCO_3$, P as KH_2PO_4. The calculated Ca and P contents include the contributions from all dietary constituents. Our analyses indicate the following Ca/P contents for the materials as used: Labco casein 0.003/0.79; hot ethanol extracted fibrin 0.2/0.11; heated egg albumen 0.04/0.084; yeast extract (Standard Brands brewers' yeast extract type 3) 0.27/3.5; Vitab (rice bran extract, Vitab type 2) 0.034/0.54.

In line with the extensive studies of Sherman and of Steenbock, 0.6% Ca has been found to be a reasonable value for normal control diets. We have only roughly explored the region between near zero content of Ca and this normal, or the effect of varying the Ca/P ratio of the diet on the antral count. In the case of studies on bone (Shohl, '36) or

on parathyroid (Stoerk and Carnes, '45) significant observations are possible over a wide range of Ca and P because both Ca and P deficiency play a role. Since even extreme P deficiency [2] does not lead to antral lesions, the Ca/P ratio is of significance only insofar as it limits the absorption of Ca which is, in this sense, specific. Table 3 shows some available data, of which the most striking feature is perhaps the persistence of the lesions up to a Ca content of 0.2%. Figure 3 is a dose-action curve

TABLE 3

Effect of Ca level (%) and Ca/P ratio on incidence and lesion count.[1]

Ca %	0.010	0.01	0.017	0.2	0.26	0.24	0.41
P %	0.044	0.43	1.89	0.24	0.42	1.24	0.45
Ca/P	0.23	0.023	0.009	0.83	0.62	0.19	0.91
Count	5.0	12.7	21.1	2.8	6.3	4.4	0.4
Incidence	18/29	238/275	11/11	20/51	21/29	4/8	0/22

[1] All of the experiments are of 4 weeks' duration and the diets contain 20 or 27% protein, adequate B complex and no vitamin D. Since the data do not represent matched animals or even contemporary experiments, and since the numbers in some groups are quite small, no precise comparison is intended. However, the simple relation between Ca content of the diet (when P is kept constant) and lesion count displayed in figure 3 indicates that the results are at least fairly consistent throughout. The lesions disappear at 0.4% Ca and are still present at around 0.2% Ca. There is a distinct effect of the Ca/P ratio which seems most marked at the lowest Ca level.

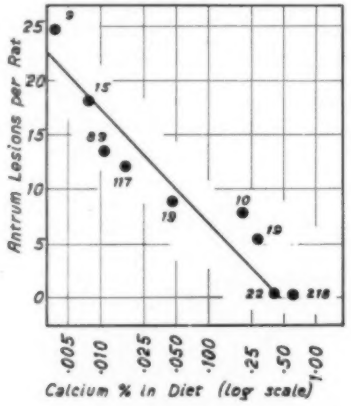

Fig. 3 Conventional dose-action curve (action = antrum count plotted against log dose = log % Ca in the diet). Numbers indicate the number of rats averaged for each point. All the diets contain 0.4 to 0.5% P and all experiments were run for 4 weeks. Differences in Ca content result from difference in added $CaCO_3$, as well as varying contributions from the other components of the diet. The data are taken from tables 3 and 6.

[2] We need not detail the results with current rickets producing diets used in vitamin D testing as well as several diets low in phosphorus and otherwise adequate, since they were uniformly negative.

for all the data on diets in which the Ca varies from 0.0037 to 0.6% and the P is kept constant between 0.4 and 0.5%: the relation between antral count and Ca content of the diet under these conditions is good enough considering the fact that the animals were not matched and the data were collected over a period of 4 years.

TABLE 4

The effect of vitamin D on lesions of the antrum.

DIET	CA%	P%	CA/P	WEIGHT (GM.) 28 days	WEIGHT (GM.) 56 days	ANTRUM Count	ANTRUM Incidence [1]	σ [2]	t	t 5% 1%
913	0.010	0.044	0.23	49	81	4.6	15/23	4.72	2.3	2.0
913 + D				49	84	1.6	6/17	2.59		2.7
905, 909	0.011	0.46	0.024	49	57	16.0	22/22	5.28	5.4	2.0
905 + D, 909 + D				50	76	5.4	13/21	7.35		2.7
1029	0.017	0.41	0.041	64	119	11.3	16/18	7.84	2.8	2.0
1029 + D				64	153	4.6	11/18	6.47		2.7
1035	0.24	1.24	0.193	60	130	4.4	4/8	6.32	1.7	2.7
1035 + D				61	163	0	0/6	0		3.0

Variance analysis of the above data.

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F	F 5% 1%
Total	132	57.0		
Experimental error	125	34.4		
Vitamin D treatment	1	1256	37	6.8
Diet (Ca/P level, ratio)	3	491	14.3	3.9
Interaction of diet and D effects	3	165	4.8	3.9

[1] See footnote 1, p. 304.

[2] σ is the standard deviation of the antrum count. The probability ratio "t" is the measure of the significance of the difference in the means for the individual comparisons; "F" is the significance measure for the variance analysis. The values which "t" or "F" must equal or exceed for the 1% or 5% levels of significance are also tabulated. (For methods, and the meaning of "t" and "F" see Snedecor, '40.) Obviously the D effect is overwhelmingly significant, the Ca/P level and ratio have very significant effects, and the extent of the D effect depends on the Ca/P level and ratio (interaction term). The serum Ca on 1029 + D (8.1 mg. per 100 ml.) is distinctly below normal although higher than the level found for 1029 (6.2).

Each comparison (with and without D) was made at the same time on rats matched for weight, sex and litter origin.

Administration of vitamin D

If the effect on the antral mucosa is due to Ca deficiency, one might look for relief of the condition when vitamin D is fed. With extreme low calcium content of the diet complete prevention cannot be expected since not enough calcium is fed for the D action to lead to much improvement of the net calcium balance. The definite existence of a D effect as shown in table 4 is further confirmatory evidence that the pertinent deficiency is in fact calcium. Incidentally, rats produced on diets containing cod liver oil have been found to be unsatisfactory subjects for this kind of study.

Neutralization of gastric acidity

In the therapeutics of gastritis and gastric ulcers in man the administration of acid binding substances, buffers and neutralizing agents has played a large role. The underlying thought is that the pathology is referable to the irritating action of the acid. A good deal of experimental work has been aimed at ascertaining whether or not HCl can be expected to have such action on living mucosal epithelium. Henning and Norpoth ('32) after an impartial review of both sides of the problem conclude that neither in their own work nor in any previous publications is there any cogent proof for either side of the argument. In view of the fact that the sole difference between some of our experimental and control diets consists of quite appreciable amounts of $CaCO_3$, it seemed worthwhile to ascertain whether other materials having equivalent neutralizing action on the gastric contents would also act as preventives. Table 5 indicates that three agents which have been used therapeutically, namely, bicarbonates, secondary phosphates, and aluminum hydroxide, have no discernible alleviating effect on antrum lesions produced in the manner here described. The phosphate apparently makes things worse. This latter result is additional evidence for a specific calcium effect.

Possible factors concerned in the hemorrhage

Our own observations as well as those of others indicate a generalized tendency to hemorrhage in calcium deficiency (Zucker and Berg, '44). Since hemorrhage is one phase of the antral pathology, attempts were made to find some intermediate processes leading to such an end result. Deficiency of ascorbic acid and vitamin K lead to hemorrhage and both of these substances are synthesized in the normal rat. The thought that calcium might be involved in either of these syntheses seemed worth

investigating. Conditions which will disturb ascorbic acid synthesis in the rat have been reported (Vedder, '38; Feng, King and Longenecker, '44). McCollum in a discussion of calcium deficiency and hemorrhage suggests "that studies should be made of the possible relationship between calcium deficiency and ascorbic acid synthesis" (McCollum et al., '39, p. 158). The possibility of inadequate ascorbic acid production as a

TABLE 5

Effect of alkalinizing agents on antral lesions.

SUPPLEMENTS TO DIET 1029 [1]	DIETARY		WEIGHT (GM.) AT			SERUM CA	ANTRUM		σ
	CA%	P%	28 days	56 days	62 days		Count	Incidence [2]	
.....	0.017	0.41	64	119		6.2	11.3	16/18	7.84
1.5% $CaCO_3$	0.62	0.41	64	194		12.0	0.06	0/18	0.24
0.8% $Al(OH)_3$	0.017	0.41	64	120		5.9	14.2	10/12	11.1
1.2% $NaHCO_3$ 1.6% $KHCO_3$	0.017	0.41	63	120		5.5	14.4	10/12	12.4
3.2% Na_2HPO_4 4.6% K_2HPO_4	0.017	1.89	63	98		5.4	21.1	11/11	7.67
1.5% $CaCO_3$, 6 day curative			63	118	147	11.5	2.1	5/12	3.17

[1] The bicarbonate is equivalent in acid neutralizing power to the $CaCO_3$. The effective neutralizing power per gram for gastric acidity of the $Al(OH)_3$ is not immediately obvious; statements on dose appear to be based on its action as a triacid base, and we have supplied it accordingly. The secondary phosphate was fed in relatively larger amounts because we wanted to get a maximal effect and knew from previous experiments that the animals would take this much. The effect of adding $CaCO_3$, either throughout or for a curative period of 6 days, is highly significant ($t = 6.1$ and 3.8, respectively). The effect of adding bicarbonate or $Al(OH)_3$ is not at all significant. The increase in lesion count due to secondary phosphate is highly significant ($t = 3.3$) and this can be ascribed to the much less favorable Ca/P ration. The two $CaCO_3$ experiments — preventive and curative — give results significantly different from each other ($t = 2.7$) indicating that a 6-day cure is incomplete.

[2] See footnote 1, p. 304.

factor in the stomach condition was tested by feeding to a group of rats on the calcium deficient diet 1029 a daily dose of 10 mg. of ascorbic acid (freshly dissolved in 0.1 ml. water). The group receiving the supplement of ascorbic acid averaged seventeen lesions per rat, which is a bit high when compared with contemporary controls. With regard to the occurrence of a secondary K deficiency both feeding of 10 mg. K_1 (2-methyl, 3-phytyl, 1,4-napthoquinone) per 100 gm. diet, and prothrombin estimations (Campbell et al., '41) were resorted to. The experimental and control groups, each consisting of nineteen rats, showed no differ-

ence in the intensity of the hemorrhage, and both antral counts were fifteen per rat. The prothrombin times for the control and K-fed animals averaged, respectively, 31.3 and 31.2 seconds; the difference was not significant. Supplementation with calcium did not affect the prothrombin time.

Other possible mechanisms of the calcium effect

Further investigations of possible mechanisms including tissue analysis are under way, but so far little progress has been made toward establishing a connection between calcium deficiency and either the hyperplasia or the hemorrhage. There are persistent indications in the cancer literature (for review, see Shear, '33) that hyperplasia of epithelial tissue is associated with a local low concentration of calcium. If some of the older interpretations are brought in line with more recent findings (Carrothers and Suntzeff, '44) many of the inconsistencies disappear. In tissue cultures, Simms ('44) found that growth of epithelial cells is favored by low-calcium and high-phosphate content in the medium, while the opposite is true for connective tissue. Interesting relations of calcium to mucus secreting glands (the rat's antrum is composed entirely of this type) have been studied by Grant ('42). The problem is not without interest when we consider that calcium deficiency leads to highly reproducible lesions in circumscribed areas limited to one region of one part of the gastrointestinal tract with hemorrhage in other regions showing up only much later and hyperplasia appearing in no other places.

EFFECT OF B DEFICIENCY

In a paper entitled "Incidence of gastric ulcer in albino rats fed diets deficient in vitamin B (B_1)," Dalldorf and Kellogg ('32) describe lesions [3] similar as to their location and identical in their histology with those which we have obtained in calcium deficiency. Such results were also reported by Sure and Thatcher ('33) and apparently by Drummond et al. ('38). Drummond's characterization of his ulcers is somewhat uncertain. We also have verified these findings (line 1, table 6, and fig. 4). The lesions are obtained in 4 weeks on diets supplemented with pure B factors other than thiamine, with autoclaved yeast (as in Dall-

[3] The meaning of "ulcer" and "erosion," the other term frequently used, is highly dependent on the school of thought to which the investigator belongs. In order to avoid arguments not pertinent to the experimental mucosal changes here under discussion, we use the term "lesion" concerning which there need by no argument. We have, however, no particular quarrel with anyone who wishes to call his lesions ulcers.

dorf's experiments), or with B complex deficiency. Dalldorf identified the lesions only microscopically and deduced their location from the histology of the surrounding tissue. In properly stretched specimens, the lesions can be seen grossly. They are neither as large nor as prominent as those in animals subjected to calcium deficiency for an equal length of time, nor is the incidence or the mean count ever as high. In all respects they resemble the appearance obtained after about 2 weeks on calcium deficiency. They are not produced by less than 4 weeks of thiamine deficiency.

TABLE 6

Effect of calcium level in B deficient diets and of thiamine level in calcium deficient diets.

CALCIUM	THIAMINE	NO. OF RATS	ANTRUM COUNT	σ	t
gm./100 gm.	*mg./100 gm.*				
0.6 or 0.8 [1]	no thiamine or no B complex	307	5.34	5.6	7.4
1.9 to 3.8 [2]		97	1.02	2.1	
0.01	0.75	47	12.7		
0.04		10	9.7		
0.0037	1.2	9	24.7		
0.01	1.5	42	14.5		
0.04		9	8.7		
0.0078	2.55	15	18.1		
0.017	4.5	117	12.1		

[1] Included in line 1 are all of the animals on diets 872, 896, 1010 and 1019.

[2] Included in line 2 are 47 rats on the autoclaved stock diet, plus rats on diets 872, 896, 1010 and 1019 with additional Ca and P. In all cases the Ca/P ratio is 1.5 to 2.0. The preventive effect of additional Ca in a B deficient diet is highly significant. The further addition of B complex (since thiamine is the single B factor whose absence leads to antrum lesions, the B complex addition is measured by thiamine content calculated from data supplied by the manufacturers of the various B concentrates) has no effect on the lesion count. The series of diets with B complex present all have a P content of 0.4 to 0.5%. The antrum count on these diets is quite definitely dependent on the Ca content, not the thiamine content.

Prevention with increased calcium

In the last week on a B deficient diet the food intake is reduced to roughly 15% of the intake for the same period of a calcium deficient diet and 10% of the intake of a normal diet; the calcium level in the diet under these conditions can be misleading as far as the relative calcium intake is concerned. It appears to be true (table 6) that added calcium supplements entirely prevent the lesions even if the food intake is limited by thiamine deficiency. On the other hand, it is seen in table 6 that

adding thiamine to a diet already adequate in this respect but deficient in calcium has no effect on the antral count.

The data show clearly that presence or absence of the disturbance in the mucosa is dependent on calcium. The reason, however, why thiamine deficiency should have an adverse effect which is mitigated by added

Fig. 4 Antral lesions (4-week B complex deficiency). The lesion defects are small pits accompanied by little or no surrounding hyperplasia. This stomach also illustrates the occurrence of fundic hemorrhage (see succeeding paper).

calcium cannot be established from the data now available. We do not know (1) whether in intermediary metabolism the calcium requirement is increased by thiamine deficiency, or (2) whether a mucosa deficient in both thiamine and calcium is more subject to attack (or less capable of carrying on healing processes) than a purely calcium-deficient one, or (3) whether the efficiency of calcium absorption is reduced by thia-

mine deficiency, or (4) whether the decrease in food intake lowers the calcium ingestion to the point where deficiency symptoms are produced. Attempts now under way to establish a separate, possibly partial, role for thiamine in the production or exacerbation of the lesions have not yet yielded conclusive results; neither is there cogent evidence that the thiamine deficiency operates exclusively through an effect on calcium, either in the total or the intermediary metabolism of this element.

A principal difficulty with further experimental work is the variability of results with B deficiency. With calcium deficiency about 90% of the several hundred individual animals show disturbances of the antrum in 4 weeks and every group gives an appreciable count. The lack of B, however, gives no such regularity of results. The individual high counts of over thirty lesions given by calcium deficiency are never reached, the overall mean is distinctly lower (about 5 against 12) and group means vary markedly including many zero values. We at first ascribed this to a seasonal effect, since for 2 successive years we obtained negligible counts during the summer months. However, it now appears that this phenomenon of refractoriness need not be seasonal.

Effect of food restriction

During the past winter paired feeding experiments with total B deficiency were carried out. The deficiency, or the food restriction in the control animals, was so severe a strain that a number of deaths occurred in both groups. Of the animals which survived the 4 weeks (nine pairs and ten extra food restriction controls), one animal on B deficiency and two on food restriction showed results in the antrum. We may have had a run of animals more refractory to antrum effects and more sensitive to the general B deficiency effects. The labor expended on each rat is considerable, partly because the danger of sudden death from starvation induced fundic hemorrhage (Zucker et al., '45) makes it necessary to feed the restricted animals (controls) twice every 24 hours.[4] Apparently in the face of this extreme variability it will require a major effort to solve the problem along these lines.

[4] Besides matters of rationale, the paired feeding experiment presents particular problems of technique when one deals with highly reduced food consumption. For instance, while the B deficient animals consume their reduced intake fairly continuously through the 24 hours, the artificially restricted controls, having been completely starved for 24 hours, with normal feeding urge madly pounce on their food (or even the hand which feeds them) and consume what they get in a few minutes. To make conditions more similar factually the allotted food is given to the control animals in divided portions. The difference between a single and divided restricted allotment has been pointed out by Mitchell (J. Biol. Chem., vol. 58, p. 918 (1923)).

STATISTICAL CONSIDERATIONS

Cowgill ('39) in a discussion of observations such as those of Dalldorf and Kellogg, states: "The failure to secure ulcers in practically every case . . . constitutes an argument in favor of the view that the ulcers arise secondarily in the course of vitamin B_1 deficiency, probably as part of the response of the stomach to a generalized systemic condition and not because the function of vitamin B_1 is specific in relation to this organ." Applying Cowgill's criteria to our 8 weeks' calcium deficiency experiments (Zucker and Berg, '44) would permit the conclusion

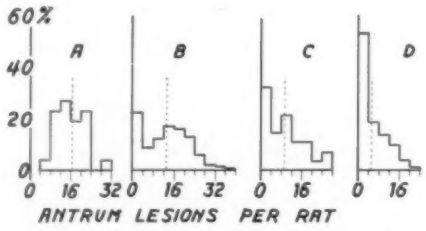

Fig. 5 The intervals on the horizontal axis represent 0–3, 4–7, etc., lesions per rat, with only the points 0, 16, and 32 numbered. The vertical axis represents the frequency in % for the various intervals on the lesion count scale. The mean in each case is indicated by the dotted line.

A: Ca deficient diet 1029 from 4 to 12 weeks of age; n = 26.

B: All Ca deficient diets series 1 and 2 from 4 to 8 weeks of age; n = 275.

C: Ca deficient diet 1029 from 4 to 6 weeks of age; n = 28.

D: B complex deficient diet 872 from 4 to 8 weeks of age; n = 234.

We have been unable to fit these curves with binomial distributions.

For the rationale of applying significance tests to data with very a-normal distribution curves (as we have done in table 6) see Treloar ('36, pp. 28, 127–128). The chief requirement for applying such tests is that the frequency distribution curve of means of a large set of groups like the ones being compared be approximately normal, and this will tend to be true even though the individual observations are quite a-normal in distribution, if the groups compared have large n values. The distribution of the means of the various experimental groups run on diet 872, with n values of 8 to 14, is slightly more normal than type B above, so groups of 97 or more should be satisfactory. A difference with a significance ratio of over 7, as in table 6, must be accepted as significant.

that the function of calcium is "specific in relation to this organ" since every one of the rats showed lesions (and this is true also of the additional animals run for 8 weeks since the appearance of last year's report). How generally this criterion can be applied is another matter. The frequency distribution graphs of figure 5 are pertinent. For four groups with diet and time variations, and having mean values of 16, 13, 9 and 5 lesions per rat, respectively, the distributions show striking differences. The similarity of the B deficient and short-time calcium

deficient groups would be in agreement with the interpretation that B deficiency is equivalent to a short-time calcium deficiency.

Distributions like D in figure 5 are held to be rare in biological data, and a distribution like B, taken by itself, would probably be interpreted as showing a mixture of two populations of inherently different susceptibility. However, taken together A, B, C, and D appear to be a series of steps representing decreasing intensity of stimulus. The least intense stimulus (D) is below the threshold for all of those more resistant individuals which spread themselves over several groups on the left side of curve A; with the sub-threshold stimulus they all pile up in the 0–3 category in graph D. No stimulus at all leads to a "distribution curve" represented by 100% in the lowest category. Presumably, if death did not intervene, a sufficiently extreme deficiency for a sufficiently long time would cause every antrum to be uniformly covered with lesions, and the distribution curve would have 100% of the rats in some limiting highest category determined by the anatomy of the rat's stomach.

We have been unable to associate the susceptibility in terms of lesion count in a 4-week experiment with anything known about the history and performance of the individual. Variance analysis of a suitable experiment involving 102 rats indicates that neither sex, initial weight nor litter origin affected the susceptibility significantly; in the case of sex there was no effect, while with initial weight and litter origin there were effects which would, if real, require many hundreds of rats to establish them as statistically significant. We nevertheless continue to match by these three criteria in setting up experiments, for whatever contribution to consistency there may be.

SUMMARY

1. Further evidence is adduced for the conclusion that in rats a rather early manifestation of calcium deficiency is found in the type of gastric lesion which occurs in the mucosa of the antrum. The changes consist of necrosis, hemorrhage and epithelial hyperplasia. Addition of phosphate accentuates the lesions, while vitamin D administration definitely reduces their number.

2. It is true, as others have found, that thiamine or total B complex deficiency will produce small lesions of the same type. However, while calcium increments in the diet abolish the lesions of thiamine deficiency, added thiamine has no preventive effect in calcium deficiency.

3. The administration of neutralizing agents as such has no effect on the lesions, indicating the improbability that gastric HCl plays a role in their formation.

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NUTRITIONAL EFFECTS ON THE GASTRIC MUCOSA OF THE RAT

II. LESIONS OF THE FUNDUS AND RUMEN [1]

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THREE FIGURES

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In the preceding paper (Zucker et al., '45) we have dealt with nutritional effects on the gastric antrum. Our original interest was principally centered on this phase of the work, the antrum being the region of the stomach most severely affected by spontaneous lesions of both man and animals. We have, however, fully recorded all the lesions produced by the rations fed; over 3000 stomachs have been examined. While a rumen is found in ruminants [2] and among laboratory animals only in the rat, mouse and hamster, the extreme hyperplasia which can be caused in the rumen of the rat by a variety of deficient diets has attracted the attention especially of cancer students. As a matter of fact the vast majority of studies involving dietary effects on the gastric epithelium have dealt with the rumen rather than the glandular portion of the stomach. The appearance of fundus and rumen lesions is illustrated in figures 1, 2 and 3.

GENERAL DISTRIBUTION OF MUCOSAL DEFECTS

The distribution of mucosal defects produced by six different rations [3] which were fed to larger groups of rats is not without interest. As can

[1] Part of this paper was reported at the 107th Am. Chem. Soc. meeting, Cleveland, 1944.

[2] The common spontaneously occurring gastric ulcers of ruminants are found not in the rumen but in the antrum. In Switzerland and Germany considerable attention has been given to this subject (for general discussion see Bongert, '12; many interesting practical details have been recorded by Tantz, '12; the pathology has been worked out by Konjetzny and Puhl, '26). Konjetzny states that during the months of February, March and April any slaughter house will afford ample material. The lesions are found in calves and young cattle which have been weaned at an early age and kept on various rations of haphazard composition. The condition has been described in the United States, but is apparently of little interest since the use of special calf meals and gruels is quite general. As to the cause the only suggestion made so far has been that of mechanical injury (straw, etc.) in spite of the fact that there is general agreement that mere trauma of the mucosa promptly heals and does not lead to ulcers. Nutritional factors obviously cannot be neglected, and, among other things, the markedly seasonal incidence suggests the involvement of calcium in analogy to the rat data presented in the preceding paper.

[3] The diets are given in table 2 of the preceding paper (Zucker, Berg and Zucker, '45).

be seen in table 1, line 1, on diet 872 which is deficient in protein and is low in B complex factors, rats will develop all three types of lesions. Of the 234 animals examined, 55% had lesions of the rumen while the fundus of 13% showed the typical hemorrhages. Antral lesions were present in 67%, with a mean count of 4.9 per rat. Lines 2 and 3 in the table refer to diets from which the calcium of the salt mixture has been

Fig. 1 The fundus is spotted with large and small adherent blood clots overlying necrotic areas, some of them arranged in streaks. The antrum and rumen are free of lesions. This is an example of severe fundic involvement. Figure 4 in the preceding paper (Zucker et al., '45) illustrates moderate involvement. The mildest form of this lesion is seen in a few pin-point hemorrhages. As against the discrete and regular appearance of the antral lesions it will be noted that the lesions of the fundus and rumen (see also figs. 2 and 3) are irregular in size and shape and thus do not permit any satisfactory counting procedure.

omitted (series 1 and 2 of the preceding paper). The first of these contains 20% air-dried cooked egg albumen or fibrin and an allowance of B factors ordinarily considered adequate. When 0.6% Ca is added (line 4), growth is good and the stomachs are normal. Without the calcium,

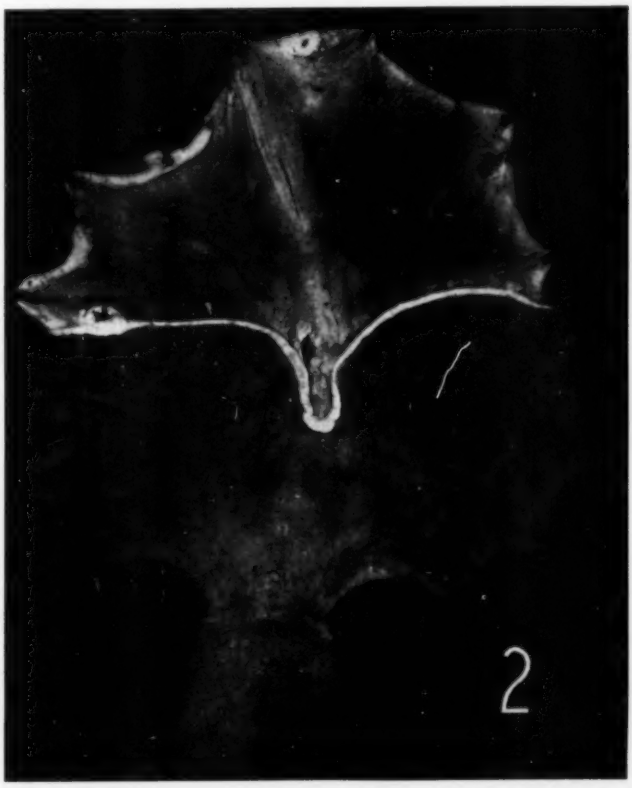

Fig. 2 Single lesions in the rumen along the ridge and at the esophageal opening, the favorite sites for this lesion. One lesion is round, has raised thickened edges and a central depression. The other at the esophagus consists of an irregular wrinkled thickening of the mucosa. The majority of the rumen lesions obtained on diets described in these papers are like those in this picture (cf. fig. 3).

however, 88% of the animals showed antral lesions with a count of 11.9 per rat. There were rumen lesions in 17% of these animals, while the fundus was normal in the sense that only one of the 134 rats showed a mild hemorrhage. The rats fed the second calcium deficient diet (line 3), which contained 27% casein and about twice as much B complex as the

previous one, showed no abnormalities except in the antrum (85% of the rats, with a lesion count of 13.6 per rat).

Among other things the data indicate that the three kinds of lesion have separate causes and that it is possible to produce any one of them in 28 days. Howes and Vivier ('36) treated their data as though lesions

Fig. 3 An unusually extensive effect involving approximately one-half of the lining of the rumen. It consists of nodular thickening with fissures and craters of irregular shapes. The ridge in this specimen shows thickening and has a beaded appearance. This antrum also shows lesions. The furrowed appearance of the fundus is normal and depends on the amount of stretching and state of contraction of the muscles.

of antrum and rumen have the same cause, and Drummond et al. ('38) thought that "32 days is probably too short a period for the appearance of gastric lesions."

FUNDUS

Apparently the only investigators who have described in detail fundic lesions produced in dietary experiments are Schiödt ('35), who attrib-

uted them specifically to B_1 deficiency, and Brunschwig and Rasmussen ('41), who obtained them under various conditions. In our experience 115 out of 366 animals (31%) on various B complex deficient diets for 4 weeks had fundic hemorrhages (this includes the 234 rats on diet 872 in table 1).

TABLE 1

Distribution of the 3 types of lesions on various diets.[1]

	DIET	NO. OF RATS	INCIDENCE			ANTRUM COUNT
			Rumen	Fundus	Antrum	
1	872, B complex deficient, 12% protein	234	128(55%)	30(13%)	156(67%)	4.9
2	Ca deficient, 20% protein	134	23(17%)	1(0.7%)	118(88%)	11.9
3	Ca deficient, 27% protein	141	2(1.4%)	2(1.4%)	120(85%)	13.6
4	Normal, 20% protein	124	1(0.8%)	0	4(4%)	0.5
5	Normal, 27% protein	94	0	0	2(2%)	0.2
6	Stock	198	2(1%)	0	2(1%)	0.2

[1] All experiments were run 4 weeks. For details on scoring the antrum lesions see the preceding paper (Zucker et al., '45).

Single B deficiencies

The question now arose which of the B factors was responsible. The feeding of a control synthetic diet (Stoerk and Zucker, '44) containing liberal allowances of the usual six factors, namely, thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid and choline, with no other known source of B factors and 27% Labco casein as the protein source, resulted in normal stomachs. Five diets, each singly deficient in one of the five factors thiamine, riboflavin, pyridoxine, pantothenic acid and choline, were fed to 4-week-old rats for a period of 4 weeks. The omission of choline led to no discernible effect on growth or any other manifestation. The other deficiencies all affected growth in varying degrees. The results on the stomach can be briefly stated to the effect that fundic lesions were associated with thiamine deficiency only. Such was the case when the respective deficiencies were complete — i.e., zero amounts of the factor in question added. With about one-twentieth of the allotment present in the complete diet no signs of hemorrhage were seen even in the case of thiamine deficiency.

Starvation

This result raised the question whether in the thiamine deficiency experiments the effect was specific for thiamine or whether the inanition

was responsible. Rats allowed the same intake of a normal diet as was spontaneously taken by B deficient animals showed a high incidence of fundic hemorrhage at the end of 3 to 4 weeks. Observations on total food deprivation show that with animals 40 ($\pm$ 1) days old a fast of 2½ days will produce definite if not fatal fundic hemorrhage with a weight loss of 30 to 40%. Attempts were made to prevent this with 1 gm. quantities daily of either cerelose or casein with or without vitamin and mineral supplements of insignificant caloric value. Twenty-five such attempts were made with no noticeable deviation from the control results except that the daily supply of 4 cal. increased survival time by about 1 day.

The trials included: 1 gm. liver powder (Wilson 1:20); 0.5 gm. yeast extract (Standard Brands type 3, equivalent to 1.5 gm. yeast); ascorbic acid (10 mg. in water given twice daily starting 3 days before food deprivation); vitamin K_1 (0.5 mg. per day) in 1 drop of oil; a mixture of 0.15 gm. Wesson salts, ascorbic acid, the six available pure B factors in amounts well above the daily requirement together with 1 gm. of either cerelose or crude casein. As a further check on the possibility that unknown factors play a role a daily allotment of 2.5 gm. fresh feces from stock rats was tried but with no effect. In animals 70 days old and weighing from 230 to 250 gm. about 5 days were required to produce a similar percentage weight loss with less but still quite definite hemorrhage in the fundus.

It appears that the fundic lesions are not related to any one essential food factor as indicated by the various trials with supplements and also by the rapidity with which the striking symptoms appear. They are probably due to a physiological process in response to absence of food, which is apparently more acute in 40 than in 70-day-old animals. It is outside the scope of this paper to look into further mechanisms which may lie in other fields such as vascular or nervous physiology.

Other combinations of several deficiencies, especially if they lead to nearly negligible food intake before the experiment is terminated, may also be responsible for fundic hemorrhage. Profuse hemorrhage in the gastro-intestinal tract has been mentioned incidentally in quite a few nutrition studies (e.g., Sherman and Derbigny, '32), where by inference at least it is related to a particular factor. Fundic hemorrhage can vary from mere petechiae to such brisk bleeding that the small intestine is filled with blood for a good part of its entire length. In these cases the source of hemorrhage is easily determined if the stomach is opened. Such massive hemorrhage may lead to sudden death, but some animals withstand it and show blood, obvious or occult, in the feces. We have

observed fundic hemorrhages in the course of another investigation where with simultaneous kidney involvement the non-protein nitrogen and urea of the blood were high. The association of high non-protein nitrogen in the blood and gastric hemorrhage is not unfamiliar in human pathology.

Effect of protein level

It is not clear why an increase in the protein level in B deficient diets was associated with an increase in the incidence of fundic lesions (12% casein, incidence 13%; 20% casein, incidence 57%; 27% casein, incidence 93%). These lesions are not produced during growth, but within a few days when weight is being lost, and it is possible that the better the animal's nutritive state was with respect to protein prior to the weight loss — i.e., the better it grew initially — the greater the lesion-producing stress when the weight loss occurs. The absence of any notation on fundic lesions in accounts of experiments involving B deficiencies and low protein (those of Sharpless, for example) may thus be accounted for.

Non-nutritional causation of fundic hemorrhage

The identical type of fundic hemorrhage can also be produced by procedures which in no way involve food or food intake level. Dodds et al. ('34) have produced acute hemorrhages which appear a few hours after the injection of pituitary extracts. These investigators emphasize that the effects are strictly limited to the fundic "acid bearing portion of the stomach" of several species including the rat. Hueper's ('27) report on gastric hemorrhages produced by Mathews and Austin in dogs by injection of parathormone shows that here also the fundic region is affected. Dr. Herbert Stoerk of this department has made similar observations on rats, the resulting disturbance being the same as that here reported in case of acute B_1 deficiency or starvation. The parathormone lesions appear long before they are seen in inanition controls.

RUMEN

The changes in the rat's rumen have for some time attracted considerable attention. Pappenheimer and Larimore ('24) reported their presence in rats fed highly deficient diets and suggested that nutritional deficiency played a role in their production. Two mutually exclusive views as to the nature of the deficiency have been advanced, one holding that vitamin A deficiency is the cause while the other relates them to

water soluble factors.[4] There can be no question regarding the finding of hyperplasia of the rumen on A-deficient diets but the conclusion that the lesions are referable to this deficiency in any specific sense can hardly be upheld: the lesions can be produced in the presence of an ample supply of vitamin A, and can be absent even though there is a well established A deficiency. However, in spite of the clear demonstration by Wolbach and Howe ('25; '33) that among the typically reproducible symptoms of vitamin A deficiency these lesions are not found, reports have continued to appear (Fridericia et al., '40; Beck and Peacock, '41) which quite plausibly relate this condition to A deficiency. In cases where the composition of the diet is given in detail it seems impossible to discern significant differences on the basis of known factors: on diets used for A assay, Richards ('35) and Bacharach and Smith ('33) recorded hyperplasia of the rumen while Dyer and Roe ('41) described the rumen as normal. Neither is it the judgment of one investigator as against that of another since Cramer ('37) failed to confirm his own previous findings and was not able to assign a cause for the discrepancy. Any final account of causation, to be satisfactory, must clarify this puzzling situation.

Sharpless has extensively studied hyperplasia of the rumen produced by deficiencies of water soluble factors in the presence of ample vitamin A. According to Sharpless the lesions are not due to a specific deficiency but occur if any one of the following factors is inadequately supplied: cystine, riboflavin, pyridoxine, nicotinic acid, and choline. It appears that Sharpless has gone further than others towards clarification of the problem.

All of our work to date has been pointed specifically at the glandular part of the stomach either antrum or fundus, but the observations on the rumen made in the course of the work are not without interest. We have been able to substantiate the findings of Sharpless on the following points: lesions are found on low casein diets, deficiency of certain B factors will produce them, and vitamin A does not prevent them even when present in more than adequate amounts.

[4] We can pass over two other suggested causes, namely, virus and worms (see Lancet, '38). Another non-nutritional cause was considered established by Büchner et al. ('28), namely, hyperacidity, on the basis of histamine injection. When Henning and Norpoth ('32) supplied the necessary control experiments, however, it was found that stomach secretion of the rat is refractory to histamine (confirmed by Friedman, '43, and by Komrow et al., '44). Brummelkamp ('38) also related the lesions to hyperacidity but did not obtain results when housing the animals in metal cages; wood must be available which the rats gnaw but do not swallow. Our studies do not resolve this point because we use only metal cages.

Prevention with purified casein

We are not convinced, however, that the non-specific causation hypothesis of Sharpless expresses the only interpretation which can be made of the data. Even in the absence of appreciable amounts of the majority of the factors each of which Sharpless holds to be essential, complete prevention can be achieved. This is illustrated in table 2 where a graded increase of purified casein in the diet from 6% to 27% progressively decreases the lesion incidence from 92% to 0%. At first sight it may seem a little incongruous with general experience that 27% of protein should be required to obtain a completely normal rumen, but on this type of diet (B complex deficient) the food intake is so low that the average daily protein ingestion is no more than on a level of

TABLE 2

Evidence that the responsible factor is present in crude and purified casein.[1]

CASEIN LEVEL	CRUDE CASEIN		LABCO CASEIN	
	No. of rats	Incidence of rumen lesions	No. of rats	Incidence of rumen lesions
		%		%
6	29	34	47	92
12	21	0	56	43[2]
20			43	8[3]
27			27	0[4]

[1] These diets contain no added source of B factors; besides the casein they have 2% fat contributing 750 units of vitamin A activity from carotene and 540 from cod liver oil, salts, and carbohydrate (starch, sucrose or cerelose) to 100. Details of diet compositions are given in table 2 of the preceding paper (Zucker et al., '45).

[2] Diet 872 — a group run at the same time as the group on the 12% crude casein diet.

[3] Diets 896 and 896 + bone ash.

[4] Diets 1019 and 1019 + bone ash.

10–12% in an otherwise adequate diet. Howes and Vivier as well as Sharpless found in a few animals that 20% of casein did not prevent the hyperplasia. This is in agreement with the data of our table 2 but we are not ready to agree with their inference that this satisfies all the conditions necessary for establishing the role of casein.

While at one time casein was almost axiomatically considered an ideal protein to use in nutrition studies, this is no longer the case. Its amino acid constitution leaves a good deal to be desired. A relative deficiency in sulfur-containing amino acids has been established, notably by the finding that growth and lactation performance can be definitely improved by supplements of cystine (Daggs et al., '35 a, b; '38; Mulford and Griffith, '42; see also Almquist, '43). Casein is difficult to purify,

as is seen when it is to be used for vitamin A studies (see account by Coward, '38). Observations with highly purified casein led a number of investigators to the conclusion that the removal of the last impurities reveals a new nutritional deficiency related to a factor closely associated with casein and present in all but the most highly purified samples (Bacharach, '33; Bacharach and Smith, '33; Coward, '38; Mapson, '32; '33). This factor can be supplied by liver extract (Mapson, '32; '33; Hartman, Dryden and Cary, '41). Jones and Gersdorff ('34), on the other hand, call attention to the fact that vigorous purification methods (alkali treatment, heat treatment) affect the casein itself in the sense that the low cystine content is still further reduced.

The difference between a sample of crude and purified casein may therefore lie either in its content of cystine (and possibly other amino acids which are labile to the method of purification) or in the amount of unidentified nutritional factors still adhering to it. In both respects the crude casein is likely to have the more favorable nutritional properties. Further study should reveal which of these is responsible for prevention of these lesions on relatively low levels of crude casein.

There will certainly also be a difference between crude and purified casein with respect to content of the known B factors, but this does not appear to be pertinent here in view of the fact that prevention is attained at a level of purified casein on which there is no difficulty in obtaining satisfactory deficiencies of all the known factors (see previous discussion under Fundus).

It may be noted here that none of the single deficiencies of the known factors including choline produced any detectable changes in the rumen in a period of 4 weeks. Neither is there as yet any indication that the 27% casein effect is equivalent to a choline (labile methyl) effect. A group of animals on a B complex deficient diet with 12% Labco casein plus an ample choline supplement showed seven out of nine animals with hyperplastic rumens, as compared with five out of eight in the control animals.

The difference between the results at a given level of feeding with crude as compared with purified casein is not a matter of difference in food intake; the pattern of growth and food intake over the 4 weeks is just about the same in the two cases. For this reason it is to be assumed that the animals on the crude casein diet are getting a larger supply of the preventive factor because there is a higher concentration of it in the crude casein; rough interpolation of the dose-action curve effects of table 2 suggests that "Labco" casein has about one-half as much of the factor as crude casein.

This point of view affords a basis for re-evaluation of the contradictory vitamin A results. Lesions of the rumen on vitamin A deficient diets may be referable to a more drastically purified casein. With a diet supplying a borderline amount of the preventive factor, any other deficiency which lowers the food intake may cause the appearance of lesions. Support for the role of lowered food intake may be found in a number of our observations of which we will cite two examples.

A supplement of 15% of autoclaved yeast added to diet 872 in no way lessened the incidence (13 out of 17 animals) and did not improve growth, while 15% of unautoclaved yeast more than doubled the food intake, changed the growth from a small net loss to a gain of 60 gm. in 4 weeks and prevented the lesions in the rumen (incidence 0 in 10 animals). The second example shows that, in the Sharpless sense, calcium could be added to the list of pertinent factors since, by comparison of lines 2 and 4 of table 1, calcium makes the difference between occurrence and prevention. The 4-week food intakes for these calcium-deficient and calcium-supplemented groups were, respectively, 140 and 230 gm.

SUMMARY

1. While fundic lesions can be produced on deficient diets, their cause probably lies in inanition rather than any specific relation of a food factor to the structure of the fundic mucosa.

2. Since the maintenance of fundic mucosa is thus removed from the narrower field of nutrition, other mechanisms involving other fields of physiology seem indicated (vascular, neurological, etc.).

3. The rat's rumen responds to some deficient diets with a quite reproducible reaction of hyperplasia and hyperkeratosis. While these lesions have been ascribed to deficiencies of both vitamin A and members of the B complex, it is shown that they can be produced in the presence of ample vitamin A in the diet, and prevented on a totally B-deficient diet by increasing the (purified) casein content to 27%.

4. The data suggest the possibility of a specific factor important for the prevention of lesions of the rumen which may be either an amino acid labile to commonly employed methods of purifying casein, or an unknown factor associated with crude casein.

5. To clarify the nature of the postulated specific deficiency the role of both food intake and diet composition will have to be subjected to more exacting study.

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THE EFFECTS OF THE DIETARY SUPPLY OF CARBOHYDRATE UPON THE RESPONSE OF THE HUMAN RESPIRATORY QUOTIENT AFTER GLUCOSE ADMINISTRATION

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The effects of the character of the preceding diet, especially with reference to fat and carbohydrate contents, upon the respiratory quotient of man has been studied by a number of workers (Frentzel and Reach, '01; Benedict, Emmes and Rich, '11; Benedict and Higgins, '12; Krogh and Lindhard, '20; Marsh, '28; Marsh and Murlin, '28; Bierring, '31; Hitchcock, '34; Sheldon, Johnson, and Newburgh, '37; Talbott, Coombs, Consolazio, and Pecora, '38). In only one of these studies (Sheldon and coworkers) was the effect of the preceding diet upon the combustion of glucose given as a dose at the beginning of the experiment determined. These workers gave diets containing 21 to 500 gm. of carbohydrates and measured the respiratory quotient (R.Q.) and the combustion of carbohydrates for 4 hours on days following these diets. In another series they measured these factors after giving 25 to 200 gm. of glucose preceded by different dietary levels of carbohydrates, but they did not make a basal determination on the day on which the glucose was given. They found that the carbohydrate level of the preceding day's diet influenced the height of the R.Q. on basal days and on the days on which glucose was given and that the effects on the carbohydrate combustion were additive, that is, the rise in basal R.Q. due to high carbohydrate intake and the rise due to carbohydrate given at the beginning of the day's measurements were summated when carbohydrates were given after high carbohydrate diets on the preceding days.

The purpose of this study was to determine the effect of the level of carbohydrates in the daily diet on the increase in the respiratory quotient (R.Q.) and on the combustion of carbohydrates after the ingestion orally of 50 gm. of glucose when the subject was in the basal condition.

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Two medical students, F. B., 26 years old, 73.0 kilos without clothes, and 179 centimeters in height, and C. J. R., 25 years old, 59.5 kilos without clothes and 173 centimeters in height, were placed on a prescribed diet for several days at definite levels of intake of carbohydrates and the basal respiratory exchange and the respiratory exchange after oral ingestion of 50 gm. of glucose was determined by the open circuit method with a Benedict (Benedict, '33) helmet and gas analysis (Carpenter, '33) in twelve successive 15-minute periods on every other day.

TABLE 1

Daily diet of F. B. and C. J. R.

DATE 1940	F. B.					C. J. R.				
	P	F	C	Cal.	R.Q.	P	F	C	Cal.	R.Q.
	gm.	gm.	gm.			gm.	gm.	gm.		
June 8	87	47	349	2167	0.91	...	...	...	....	...
June 9	87	47	349	2167	0.91	87	47	349	2167	0.91
June 10	75	63	344 [1]	2243	0.89	87	47	349	2167	0.91
June 11	91	70	349	2475	0.89	69	57	335 [1]	2129	0.90
June 12	85	75	335 [1]	2355	0.88	87	47	349	2167	0.91
June 13	87	63	329	2231	0.89	73	81	335 [1]	2361	0.88
June 14	[2]	[2]	[2, 1]	[2]	[2]	87	73	349	2401	0.89
June 15	85	185	87	2353	0.76	[2]	[2]	[2, 1]	[2]	[2]
June 16	83	185	75	2297	0.76	83	197	75	2405	0.76
June 17	108	174	77 [1]	2306	0.77	83	197	75	2405	0.76
June 18	89	191	84	2411	0.76	107	186	76 [1]	2406	0.76
June 19	108	174	77 [1]	2306	0.77	83	197	75	2405	0.76
June 20	83	185	75	2297	0.76	107	186	76 [1]	2406	0.76
June 21	108	178	50 [1]	2234	0.75	83	197	75	2405	0.76
June 22	107	178	11	2074	0.74	108	187	50 [1]	2315	0.75
June 23	107	178	11	2074	0.74	110	184	11	2140	0.74
June 24	108	178	50 [1]	2234	0.75	110	184	11	2140	0.74
June 25	...	...	...	....	...	108	187	50 [1]	2315	0.75
June 26	...	...	...	....	...	107	184	0	2084	0.73

[1] Fifty gm. glucose instead of breakfast.

[2] Diet for the day chosen by the subject. No record made.

The amounts of protein, fat, carbohydrates, calories, and the calculated R.Q.'s in the daily diet are given in table 1. The theoretical R.Q. of the dietary intake was calculated from the usual factors for respiratory exchange on the assumption that the fat and a mixture of carbohydrates (starch and sugar) was completely oxidized and that the protein gave 774 ml. of carbon dioxide and required 957 ml. of oxygen per gram for the portion of the protein burned (Carpenter, '39). There were three levels of carbohydrates in the diets namely, approximately 350 gm., 75 gm., and 11 gm., respectively. Table 1 included the 50 gm. of glucose on

the days on which this amount was given. The R.Q.'s of the diets calculated from the components show that at the high level, the R.Q.'s varied from 0.88 to 0.91 and at the 75-gm. level, from 0.76 to 0.77. The lowest R.Q. of the diet, 0.73, was on June 26, with C. J. R., with no carbohydrates in the diet.

The body weights of the subjects fell from 73.2, with F. B., to 70.0 kilos and with C. J. R., from 59.1 to 57.4 during the series. They continued their physical activity in hospital wards and medical school throughout the period.

TABLE 2

Analyses of blood and urine.[1]

DATE AND SUBJECT	CARBOHYDRATE LEVEL	TIME	URINE SUGAR	ACETONE	DIACETIC ACID	BLOOD SUGAR
	gm.		%			%
F. B.						
June 10	350	Fasting	0	—	0	0.09
		After sugar	0.2	—	—	0.07
June 21	75	Fasting	tr	0	0	0.09
		After sugar	0.3	tr	0	0.07
June 24	11	Fasting	0.3	tr	0	0.07
		After sugar	0.4	+	0	0.06
C. J. R.						
June 15	350	Fasting	0	—	0	0.09
		After sugar	0	—	0	0.07
June 22	75	Fasting	0.2	—	0	0.08
		After sugar	0	—	0	0.07
June 25	11	Fasting	0	tr	0	0.09
		After sugar	0.4	+	0	0.09
June 27	0	Fasting	0	+	0	0.08
		After sugar	0	tr	0	0.09

[1] Where dashes occur in the table, no determinations were made.

The urines were collected immediately on arrival at the Laboratory in the morning and then immediately after the series of determinations on the R.Q.'s were made after sugar. Blood samples were also taken about one-half hour after the first urine was voided and at the end of the experimental periods at about 12:30 P.M. The urines were tested for sugar, acetone, and diacetic acid in most cases, and the blood sugar was determined, but unfortunately the urinary nitrogen was not determined. Sample results at the different levels of carbohydrate intake in which the amounts given represent the diet of the day before the test are given in table 2. The striking fact is that with the marked changes in carbohydrate there were practically no changes in the blood sugar. The lowest level is with F. B. on June 24 when it fell to 0.06% in the sample after

the sugar was taken. Usually, the blood sugar was lower at noon than in the fasting condition by 0.01 to 0.02%. When the carbohydrate intake was reduced to only 11 gm. the administration of glucose was not followed by as marked a fall in the blood sugar with C. J. R. as previously. It may be supposed that with a low carbohydrate intake less insulin was made and there was a slighter response by the outpouring of insulin after giving the 50 gm. of glucose than was true on the higher diet. Urine analyses were for the most part negative and acetone appeared only in traces in the case of F. B. on June 21 and June 24 and similarly on June 25 and June 27 with C. J. R. Nothing resembling clinical acidosis occurred even when the subjects were on only 11 gm. carbohydrate and 197 gm. fat.

The changes in the R.Q. from the base line for 3 hours after the ingestion of 50 gm. of glucose are given in table 3. With F. B., the base line's R.Q.'s after 350 gm. of carbohydrates were much lower than the calculated R.Q. of the diet so that from the time the last meal was ingested until the base line measurement was made, there was a marked fall. This fall may be due to the increased combustion of body fat as the diet was inadequate as shown by the fact that both subjects lost weight during these series of observations. The calculated R.Q. of the diet does not imply that that is necessarily the expected basal R.Q., but was made to show a comparison between the calculated R.Q.'s of the diet and those actually found in the basal condition.

The ingestion of 50 gm. of glucose was followed by a marked rise in R.Q. for the entire 3 hours with an average maximum rise of 0.10 in the seventh and eighth 15-minute periods. The net average increases in R.Q. for the 3 hours were 0.08 and 0.06 for the 3 days on the 350-gm. level of carbohydrate intake. When F. B. was on 75 to 87 gm. level in the daily diet, the base line R.Q.'s were nearly the same as the calculated R.Q.'s of daily diet. All three experiments with 50 gm. of glucose showed rises in R.Q. but not so large as with the 350 gm. level of dietary carbohydrates. The net average rise for 3 hours for each of the 3 days was 0.04, thus definitely lower than when the subject was on 350 gm. level of dietary carbohydrate intake. On June 24, after 2 days with 11 gm. of carbohydrates, the base line R.Q. was 0.72, but in spite of this, the rise in R. Q. after ingestion of 50 gm. of glucose was as high as when the subject was on the 75 gm. daily carbohydrate level. The net average 3-hour increase was 0.05.

With C. J. R., the general findings were not so definite. The base line R.Q.'s were not so low and on the 75 gm. carbohydrate daily dietary level, the base line R.Q.'s were higher on 2 days than those calculated

TABLE 3

Changes in the respiratory quotient after oral ingestion of 50 gm. of glucose as affected by the preceding diet.

DATE AND CONDITION 1940	BASAL R.Q.	CHANGES FROM BASAL IN R.Q. (15-MINUTE PERIODS).											
		1	2	3	4	5	6	7	8	9	10	11	12
F. B.													
350 gm. carbohydrate													
June 10	0.77	− 0.01	+ 0.03	+ 0.07	+ 0.09	+ 0.11	+ 0.10	+ 0.12	+ 0.13	+ 0.11	+ 0.10	+ 0.02	+ 0.08
June 12	0.80	− 0.01	+ 0.01	+ 0.08	+ 0.09	+ 0.08	+ 0.07	+ 0.09	+ 0.09	+ 0.08	+ 0.08	+ 0.03	+ 0.06
June 14	0.79	− 0.03	+ 0.02	+ 0.08	+ 0.10	+ 0.08	+ 0.09	+ 0.09	+ 0.07	+ 0.07	+ 0.06	+ 0.05	+ 0.01
75 gm. carbohydrate													
June 17	0.76	− 0.02	− 0.01	+ 0.03	+ 0.04	+ 0.04	+ 0.07	+ 0.08	+ 0.08	+ 0.08	+ 0.03	+ 0.04	+ 0.05
June 19	0.77	− 0.02	− 0.01	+ 0.05	+ 0.06	+ 0.05	+ 0.05	+ 0.08	+ 0.08	+ 0.04	+ 0.03	+ 0.02	+ 0.03
June 21	0.75	− 0.01	+ 0.01	+ 0.02	+ 0.04	+ 0.03	+ 0.05	+ 0.09	+ 0.07	+ 0.06	+ 0.05	+ 0.07	+ 0.02
0–11 gm. carbohydrate													
June 24	0.72	− 0.01	+ 0.01	+ 0.03	+ 0.02	+ 0.06	+ 0.08	+ 0.08	+ 0.08	+ 0.09	+ 0.06	+ 0.08	+ 0.04
C. J. R.													
350 gm. carbohydrate													
June 11	0.84	− 0.05	− 0.01	+ 0.02	+ 0.05	+ 0.05	+ 0.07	+ 0.10	+ 0.08	+ 0.05	+ 0.07	+ 0.07	+ 0.09
June 13	0.80	− 0.01	+ 0.02	+ 0.07	+ 0.05	+ 0.05	+ 0.05	+ 0.10	+ 0.05	+ 0.09	+ 0.03	+ 0.03	+ 0.07
June 15	0.82	− 0.04	+ 0.01	+ 0.04	+ 0.06	+ 0.06	+ 0.06	+ 0.04	+ 0.09	+ 0.07	+ 0.07	+ 0.05	+ 0.07
75 gm. carbohydrate													
June 18	0.75	− 0.02	+ 0.02	+ 0.04	+ 0.05	+ 0.05	+ 0.05	+ 0.07	+ 0.08	+ 0.08	+ 0.07	+ 0.07	+ 0.08
June 20	0.78	− 0.02	− 0.03	0.00	+ 0.01	+ 0.01	+ 0.04	+ 0.03	+ 0.06	+ 0.06	+ 0.06	+ 0.01	+ 0.01
June 22	0.78	− 0.03	− 0.01	0.00	+ 0.01	+ 0.03	+ 0.04	+ 0.06	+ 0.06	+ 0.05	+ 0.03	+ 0.06	+ 0.03
0–11 gm. carbohydrate													
June 25	0.75	0.00	− 0.01	+ 0.02	0.00	+ 0.02	+ 0.05	+ 0.04	+ 0.07	+ 0.06	+ 0.04	+ 0.05	+ 0.08
June 27	0.74	− 0.03	− 0.01	0.00	+ 0.03	0.00	+ 0.02	+ 0.03	+ 0.05	0.00	+ 0.03	+ 0.01	+ 0.06

from dietary intake. The net average rise in the R.Q. for 3 hours on each of the 3 days on the 350 gm. carbohydrate intake was 0.05 and on the 3 days with 75 gm. for dietary carbohydrate, the net average rises for 3 hours in R. Q. were 0.05, 0.02, and 0.03, and on the lowest carbohydrate intake, were 0.04 and 0.02.

The increases in the heat production and in the combustion of carbohydrates are given in table 4. The heat production in the base line

TABLE 4

Increase in heat production and combustion of carbohydrates after ingestion of 50 gm. of glucose (values for 3 hours).

DATE 1940	BASE LINE		INCREASE AFTER 50 GM. GLUCOSE	
	Heat production	Carbohydrate burned	Heat production	Carbohydrate burned
	cal.	*gm.*	*cal.*	*gm.*
F. B.				
350 gm. carbohydrate				
June 10	229.6	8.1	13.8	16.3
June 12	237.8	15.2	6.3	12.8
June 14	229.7	12.7	19.5	13.0
75 gm. carbohydrate				
June 17	215.4	5.9	25.2	9.1
June 19	226.7	7.1	9.1	8.9
June 21	236.4	5.4	3.2	8.1
11 gm. carbohydrate				
June 24	220.0	0.0	13.5	7.9
C. J. R.				
350 gm. carbohydrate				
June 11	182.8	16.1	12.8	9.0
June 13	184.1	10.2	15.9	9.2
June 15	186.4	14.0	5.9	7.8
75 gm. carbohydrate				
June 18	202.3	4.4	3.3	8.6
June 20	190.8	8.8	12.9	4.0
June 22	191.7	8.1	9.2	5.1
0–11 gm. carbohydrate				
June 25	194.8	2.5	8.8	6.2
June 27	188.8	1.6	16.6	3.4

periods of F. B. on the 350 gm. intake of carbohydrates averaged 232.4 cal., on the 75 gm., 226.2, and on the lowest, on 1 day, 220.0 cal. There is no evidence with this subject that the varying levels of carbohydrate intake affected the base line heat production. The increase in heat production due to the ingestion of 50 gm. of glucose averaged 13.2, 12.5, and 13.5 cal. for the three levels, but there is considerable variation so that this agreement is only fortuitous. With C. J. R., the average base

line heat production with 350 gm. carbohydrate intake was 184.4, and with the 75 gm., 194.9 calories, and this difference is significant. Part of this increase may have been due to a higher protein level of the diet on the days preceding the measurements, although no data on the nitrogen elimination in the urine are available. On the lowest level it was 191.8. This average is not considered as a significant increase from that on the 350 gm. level. The average increases in the heat production due to 50 gm. of glucose were 11.5, 8.5, and 12.7 cal. in the three series with considerable variation in the individual values.

The combusion of carbohydrates was calculated in the usual empirical manner from the respiratory exchange and an assumed combustion of protein. The protein combustion was calculated from the amount of protein in the preceding day's diet and a coefficient of digestibility of 90%. An actual determination of the nitrogen elimination in the urine would have been better, but it is believed that for comparative purposes the above assumption is justifiable. That changes in carbohydrate combustion after glucose ingestion represent increased carbohydrate combustion is shown by a recent study (Edwards, Bensley, Dill and Carpenter, '44) in which the lactic acid and the alveolar carbon dioxide were determined before and after the ingestion of glucose in man.

The base line carbohydrate combustion with F. B. averaged in the three series 12.0, 6.1, and 0 gm. for the 3 hours. There was thus a definite effect of the carbohydrate intake of the preceding day upon the carbohydrate combustion in the base line condition. After ingestion of 50 gm. of glucose there was an average increase during the 3 hours of 14.0, 8.7, and 7.9 in the three different types of experiments. Thus, the shift from the level of 350 gm. of carbohydrate to one of 75 gm. on the preceding day lowered the increase in carbohydrate combustion for 3 hours following ingestion of 50 gm. of glucose by 5.3 gm. When little or no carbohydrate was taken on the preceding day, there was still an increase of 7.9 gm. in the 3 hours following ingestion of 50 gm. of glucose. The base line carbohydrate combustion of C. J. R. for the 3 hours was 13.4 when on the 350 gm. of carbohydrate level, 7.1 gm. on the 75 gm. level, and 2.1 on the 0 to 11 gm. level. The difference between the first two series, 6.3 with C. J. R., was nearly the same as with F. B., 5.9 gm. This subject showed in all three series the effect of the preceding day's diet on the carbohydrate combustion in the basal condition on the following morning. The increase in the combustion of carbohydrates for 3 hours with this subject after the ingestion of 50 gm. of glucose was 8.7 gm. on the 350 gm. dietary level, 5.9 on the 75 gm. level, and 4.8 on the 0 to 11 gm. level. The differences in the increases in carbohydrate combustion

after 50 gm. of glucose were not so large when C. J. R. was at the three different levels of dietary carbohydrate intake as with F. B. C. J. R. was lighter in weight and had a lower metabolism than F. B. and therefore, C. J. R.'s need of carbohydrate was less than that of F. B. When C. J. R. was on the 350 gm. dietary level, two of the three base line R.Q.'s were higher than those of F. B., but in spite of this, the increase in carbohydrate combustion was not so great with C. J. R. as with F. B. Judging from these two subjects, one may suggest that the amount of increase in carbohydrate combustion after 50 gm. of glucose varies with individuals and that given a certain basal level in one subject, one may not expect the same rise after 50 gm. of glucose in another individual with the same basal level of carbohydrate combustion. It does not follow that the subject who has the highest basal carbohydrate combustion will have the greatest increase in carbohydrate combustion after ingestion of glucose. The level of basal carbohydrate combustion does affect the increase in carbohydrate combustion after the ingestion of glucose but even when the basal combustion reaches zero, the normal individual is still able to burn some carbohydrate in spite of the need to augment the glycogen supply. One might expect that with so drastic a lowering of carbohydrate intake with undiminished physical activity the need for deposition of glycogen would be so great that all the glucose would be laid down as glycogen and none would be burned. It is apparent that mere reduction of glycogen does not prevent oxidation of carbohydrate when, as in the normal individual, abundant insulin is present.

SUMMARY

Two medical students were placed on diets for several days at three levels of intake of carbohydrates, namely approximately 350, 75 and 0 — 11 gm. per day, respectively. The respiratory exchange was measured on alternate days in the basal condition and for 3 hours after the ingestion of 50 gm. of glucose. The combustion of carbohydrates was calculated in the usual empirical manner from the respiratory exchange and the protein level of the day's diet preceding the determinations of the respiratory exchange.

The basal combustion of carbohydrates calculated to 3 hours averaged 12.0, 6.1, and 0 gm. for one subject, and 13.4, 7.1, and 2.1 gm. for the other subject for the three dietary levels.

The increase in combustion of carbohydrates after the ingestion of 50 gm. of glucose with one subject was 14.0, 8.7 and 7.9 gm. and for the other subject, 8.7, 5.9 and 4.8 at the three dietary carbohydrate levels.

Thus the level of carbohydrates in the preceding diet affected the basal combustion of carbohydrates noticeably, and to some extent the increases in carbohydrate combustion after ingestion of glucose, but not so much as one would expect when the demand for replacement of glycogen supply is considered.

The respiratory exchange measurements were made by Mr. Basil James and the diets were under the supervision of Miss Rosina Vance.

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BODILY STORAGE OF VITAMIN A IN RELATION TO DIET AND AGE, STUDIED BY THE ASSAY METHOD OF SINGLE FEEDINGS [1]

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Independent investigations in several laboratories have shown that vitamin A has important relations to nutritional wellbeing at all ages and that the body has powers, when its nutritional intake is ample, to lay up in the liver considerable stores of this vitamin for future use. The experiments recorded in this and the accompanying paper (Caldwell, MacLeod, and Sherman, '45) were designed to develop the quantitative knowledge of this storage function.

PLAN AND METHODS

The plan was to feed well matched young rats, of like hereditary and nutritional backgrounds, from the age of 28 days upon diets, alike in other respects, but of three levels of vitamin A value, namely, 3, 6, and 12 International Units, respectively, of vitamin A per gram of air-dry food. These levels may also be stated as 0.8, 1.6, and 3.2 I.U. of vitamin A, respectively, per Calorie of food. (The laboratory record numbers of these three diets are Nos. 16, 360, and 361, respectively.) The extra vitamin A was introduced in the form of highly potent codliver oil. The offspring of these original experimental animals were continued on their respective family diets until killed for determination of the vitamin A contents of their livers at the ages of 30, 60, 90, 150, 225, and 300 days.

In this study liver storage is believed to be sufficiently indicative of bodily storage, because previous investigations in other laboratories as well as our own have shown the quantities stored elsewhere to be negligible compared with those found in the liver (Sherman and Boynton, '25; Baumann, Riising, and Steenbock, '34; Kao and Sherman, '40; Little, Thomas, and Sherman, '43; Rohrer and Sherman, '43.)

[1] Aided by grants from the Carnegie Institution of Washington and from The Nutrition Foundation, Inc.

TABLE 1

Vitamin A per gram of liver.

AGE	DIET 16 (WITH 3 I.U./GM.)		DIET 360 (WITH 6 I.U./GM.)		DIET 361 (WITH 12 I.U./GM.)	
	Males	Females	Males	Females	Males	Females
28 days						
Mean ± its P.E.[1]	5.7 ± 1.23	4.7 ± 1.17	44.8 ± 3.15	34.3 ± 2.77	157.3 ± 19.32	144.7 ± 13.27
C.V.[2]	117	146	35	42	37	43
Number of cases	13	15	11	12	4	10
60 days						
Mean ± its P.E.	6.8 ± 1.53	5.7 ± 2.02	71.3 ± 6.74	71.7 ± 5.16	177.3 ± 9.21	204.3 ± 8.07
C.V.	126	184	47	40	27	21
Number of cases	14	12	11	14	12	12
90 days						
Mean ± its P.E.	2.7 ± 0.57	8.7 ± 1.05	64.8 ± 2.33	77.0 ± 5.19	244.0 ± 6.12	300.5 ± 6.71
C.V.	114	60	18	38	13	12
Number of cases	13	11	11	14	12	12
150 days						
Mean ± its P.E.	10.8 ± 1.40	19.8 ± 2.24	115.2 ± 4.61	139.1 ± 6.43	379.4 ± 12.67	472.1 ± 15.06
C.V.	67	57	23	26	18	17
Number of cases	12	11	14	14	13	13
225 days						
Mean ± its P.E.	2.1 ± 0.77	4.0 ± 1.14	133.8 ± 5.07	183.8 ± 7.78	604.6 ± 18.29	751.7 ± 26.07
C.V.	198	142	20	22	15	18
Number of cases	13	11	12	12	11	12
300 days						
Mean ± its P.E.	2.1 ± 0.49	13.9 ± 2.33	189.5 ± 14.53	253.5 ± 15.69	783.5 ± 36.67	838.1 ± 42.14
C.V.	122	91	38	35	24	25
Number of cases	12	13	11	14	12	12

[1] Probable error.

[2] Coefficient of variation.

The sexes were caged separately from the age of 28 days. Livers were removed from the experimentally-fed animals and sampled for assay in the manner described in the accompanying paper (Caldwell, MacLeod, and Sherman, '45), and their vitamin A values determined by the single-feeding method developed by Todhunter (Sherman and Todhunter, '34), with separate male and female controls.

EXPERIMENTAL DATA

Table 1 shows the vitamin A values in International Units per gram of liver, averaged separately by age and by sex, each mean value accompanied by its probable error (P.E.), coefficient of variation (C.V.), and number of cases.

TABLE 2

Vitamin A found in livers of rats (from Diets 360 and 361) expressed as approximate percentages of the total potential surpluses which they had received in their food.

AGE	ON DIET WITH 6 UNITS PER GM.		ON DIET WITH 12 UNITS PER GM.	
	Males	Females	Males	Females
60 days	23.0	22.8	28.8	29.5
90 days	10.7	10.5	20.8	21.1
150 days	8.8	10.2	15.8	17.6
225 days	6.2	7.8	15.0	16.3
300 days	7.0	8.9	12.6	13.2

The extremely small amounts found in the livers of the rats whose food contained 3 units per gram are probably best considered as approximately the minimal amounts or concentrations essentially involved in normal maintenance processes. We may consider that this level of nutritional intake supplies approximately the actual (or minimal normal) needs but provides practically no reserve surplus. This may be used as a base-line for an approximate calculation of the quantitative relations of the surpluses offered by the diets of richer vitamin A content and the amounts of stored (reserve) surplus found at different ages in the livers of the animals which had received these diets. The results of these calculations are shown in table 2.

DISCUSSION

Although some observations and discussions tend to throw emphasis upon the fact that surpluses received by the body are not always taken up quickly by the liver (Review, '43), the experiments here reported show that the liver continues over at least a good proportion of the normal life cycle to store a very significant part of surpluses furnished

by intakes two and four times as liberal as the commonly recommended allowances for normal nutrition.

As explained in the preceding section, a level of intake of 3 I.U. of vitamin A per gram of air-dry food, or 0.8 I.U. per Calorie, sufficed for normal growth and maintenance but not to provide for any significant reserve store. The amounts found in the livers of healthy rats on this level at all ages from end of infancy to middle age showed very high coefficients of variation (57 to 198) but this is chiefly because the actual concentrations were so small as to be practically negligible (usually less than 10 I.U. per gram).

At a level of intake twice as high (1.6 I.U. per Calorie) the coefficients of variation were of the order of 20 to 40. With this greater regularity of results, it is doubtless significant that at this level of intake the body increases its reserve store of vitamin A from the end of infancy to middle age, — from 28–30 days to 300 days in the rat. This trend, however, is not quantitatively uniform. The stores found at the age of 90 days show in the females a slackening in the otherwise progressive rate, and in the males a temporary recession; both sexes, however, showing resumption of the upward trend before the age of 150 days. In the experiments of Little who used an entirely different assay method, the data while fewer in number also show a slackening of the storage trend around the age-range of 90 to 150 days (Little, Thomas, and Sherman, '43). As a similar slackening of storage at about 90 days on this same level of intake appears also in the data found simultaneously by a third method of assay (Caldwell, MacLeod, and Sherman, '45), the finding, although not striking, is probably significant. Its significance appears to be that in the age range of adolescence and young adulthood the body has a relatively high vitamin A requirement, so that the intake level of 1.6 I.U. per Calorie of food affords a smaller margin than in the earlier and later age periods.

Consistently with this view, when the level of intake is again twice as high (3.2 I.U. per Calorie of food) the potential surplus is so abundant that even in the age period of relatively higher requirement the body receives enough from the food to permit an uninterrupted trend of increasing reserve storage. This bodily storage, while always higher on the higher level of intake, is not so large as to account quantitatively for the whole difference of vitamin A content of the food consumed. From table 2 it may be seen that of the arithmetically potential surplus furnished by the food consumed, only about one-tenth to three-tenths was recovered as increase of the liver store.

With the moderate surpluses furnished by the experimental diets here used, the larger intake in food led to larger storage in the body both in absolute amount and when reckoned as percentage of the potential amount ingested. Of very much larger intakes only smaller percentages are stored; but these small percentages may mean large numbers of units, or bodily stores sufficient to meet the body's nutritional needs for a relatively long time (Bessey and Wolbach, '38; Moore, '37; Pett, '39; Ralli, Papper, and Baumann, '41; Sherman and Cammack, '26.)

For both the levels of intake here discussed it appears from the data in table 2, that bodily storage was most efficient in the earliest period of liberal vitamin A diet and later settled down to the storage of a lesser proportion as the storage capacity approached saturation or equilibrium with intake.

The general order of magnitude of the storages of vitamin A per gram of liver here found in rats are comparable with those observed in human livers by Ralli and coworkers ('41).

In the rats that were kept throughout their natural lives on the same three diets compared in the present paper, it was observed that the higher intake levels, which result in higher levels of bodily storage of vitamin A result also in more favorable life histories (Sherman, Campbell, Udiljak, and Yarmolinsky, '45.)

SUMMARY

In experiments with rats, a diet furnishing 3 I.U. of vitamin A per gram of air-dry food (0.8 I.U. per Calorie) has supported growth and life histories that are within the normal range but has resulted in the storage of only negligible reserves in the liver.

Parallel animals on diets containing, respectively, twice and four times as much vitamin A, acquired larger stores (and higher with the higher intake level) both per gram of liver and in total amount.

At the highest of these levels the body store increased steadily throughout the age range of the experiment (28 to 300 days). At the intermediate level there was a slackening of the storage trend at about 90 days, as if requirement were greatest at about that age.

In no case were differences in storage as great as if all the theoretically potential surplus had been stored, the actual efficiency of storage being of the order of 10 to 30% of the theoretically available surplus ingested in the food.

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BODILY STORAGE OF VITAMIN A IN RELATION TO DIET AND AGE STUDIED BY MEANS OF THE ANTIMONY TRICHLORIDE REACTION USING A PHOTO-ELECTRIC COLORIMETER [1]

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The earlier observations and experiments upon the importance of bodily storage as a factor in the nutritional significance of vitamin A were reviewed by Sherman and Cammack ('26) who studied quantitatively the effects of different levels and durations of preparatory feeding upon the storage of vitamin A in the body as judged by the survival period after transfer to vitamin A-free diet. In their experiments (with rats) they found, that the bodily store of vitamin A was promptly and markedly increased by the addition of cod liver oil to an already good diet but that the body only gradually approaches the attainment of its maximum store.

Subsequent development of methods for direct assay of the vitamin A content of tissues, and the finding of Baumann, Riising, and Steenbock ('34) that the assay of liver tissue alone is sufficient to indicate differences in the body store of this vitamin in well nourished animals, have facilitated further studies in this field. Lewis, Bodansky, Falk, and McGuire ('42) have compared the concentration of vitamin A in the blood with the storage in the liver as measured by the antimony trichloride colorimetric method. Using a spectrophotometric technique, Little, Thomas, and Sherman ('43) compared the concentrations of vitamin A in the livers of rats that had been fed diets of three levels of vitamin A value up to ages of 30, 90, and 150 days; while Rohrer and Sherman ('43), using the Todhunter method of assaying by a single-feeding technique, compared rats from three diets at the ages of 30 and 60 days. These last-mentioned investigations confirmed the importance of the level of intake but also indicated the desirability of more comprehensive studies to differentiate more clearly and conclusively

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the respective relations of bodily storage and nutritional need at different ages as well as at different levels of intake.

The present paper reports the results of one series of such studies making use of an adaptation of the antimony trichloride method of assaying liver tissue for its vitamin A content. A simultaneous series in which the Todhunter method was employed is being reported separately.

EXPERIMENTAL

The experimental animals were rats of known hereditary and nutritional background, all of the same (Osborne-Mendel albino) strain, inbred in this laboratory through more than twenty generations. Different groups of these animals were fed in parallel on the following three diets: (1) Diet 16, consisting of five-sixths ground whole wheat and one-sixth dried whole milk, with 2% as much sodium chloride as wheat, the air-dry food mixture having a vitamin A value of 3 International Units per gram; (2) Diet 360, the same except for the addition of enough high-grade cod liver oil to bring the vitamin A value to 6 I.U. per gram; (3) Diet 361, the same with additional cod liver oil to bring the vitamin A value of this diet to 12 I.U. per gram. The amount of cod liver oil needed to thus fortify diets 360 and 361 was too small to affect significantly the composition and energy value of the diet or the intake of water-soluble vitamins. The food mixture and distilled water were available to each animal at all times.

Healthy, typical offspring of families that were in at least the second generation of their respective diets were killed at the ages of 30, 60, 90, 150, and 300 days and the liver tissue assayed by the antimony trichloride method as modified by Dann and Evelyn ('38) and by Lewis et al. ('42), using a photoelectric colorimeter (Coleman spectrophotometer) to measure the intensity of the transmitted light.

Table 1 shows for males and females, the average vitamin A content per gram of liver tissue at each of the five stated ages for each of the three diets, together with the probable errors of the means, the coefficients of variation, and the number of cases. While the females tend to store slightly higher concentrations of vitamin A than males of the same age on the same diet, the relationships of storage to level of intake and to age are essentially similar for the two sexes.

Table 2 shows correspondingly the averages for the same animals given in terms of International Units in the total liver. The larger size of the males (and their livers) approximately balances the higher concentration of vitamin A in the livers of the females.

TABLE 1

Vitamin A content per gram of liver tissue in rats.

AGE	DIET 16 (WITH 3 I.U./GM.)		DIET 360 (WITH 6 I.U./GM.)		DIET 361 (WITH 12 I.U./GM.)	
	Males	Females	Males	Females	Males	Females
30 days						
Mean ± its P.E.[1]	3. ± 1.0	2. ± 0.3	34. ± 2.1	31. ± 1.5	69. ± 3.0	87. ± 3.2
C.V.[2]	146	77	29	23	20	17
Number of cases	9	11	10	10	10	10
60 days						
Mean ± its P.E.	1. ± 0.2	2. ± 0.2	68. ± 3.2	73. ± 3.4	163. ± 5.3	205. ± 8.6
C.V.	93	63	27	22	15	20
Number of cases	16	12	15	10	10	10
90 days						
Mean ± its P.E.	1. ± 0.1	2. ± 0.1	51. ± 2.3	90. ± 5.0	244. ± 12.1	335. ± 14.2
C.V.	105	31	21	26	23	20
Number of cases	11	10	10	10	10	10
150 days						
Mean ± its P.E.	2. ± 0.1	10. ± 4.1	129. ± 5.7	193. ± 7.2	525. ± 27.9	680. ± 49.5
C.V.	47	240	21	18	25	34
Number of cases	11	14	10	11	10	10
300 days						
Mean ± its P.E.	0.1 ± 0.07	10. ± 2.9	226. ± 15.4	291. ± 20.8	1193. ± 41.0	1547. ± 32.7
C.V.	400	168	30	42	18	9
Number of cases	16	14	9	16	13	9

[1] Probable error. [2] Coefficient of variation.

TABLE 2

Vitamin A contents of total livers of rats: International Units.

AT THE AGE OF	DIET 16 (with 3 I.U./gm.)		DIET 360 (with 6 I.U./gm.)		DIET 361 (with 12 I.U./gm.)	
	Males	*Females*	*Males*	*Females*	*Males*	*Females*
30 days	7	3	73	68	159	205
60 days	4	6	445	425	1165	1111
90 days	4	10	388	525	1905	2110
150 days	12	57	1162	1190	4424	3776
300 days	1	65	2167	1862	11453	9546

DISCUSSION OF RESULTS

Inasmuch as the rats were all of the same strain and each from a family that had been for at least a generation on the same diet as the respective experimental animal, it is noteworthy that even at the age of 30 days (end of infancy in the rat) the effects of the family diet are already significant in the concentrations and amounts of liver vitamin A.

On the diet with 3 I.U. of vitamin A value per gram of air-dry food or about 0.8 I.U. per Calorie the amount of storage of vitamin A in the liver at any of the five age levels investigated was so small as probably to be only negligible, though there might be considered to be a slight upward trend with maturity in the females but not in the males.

On the diet twice as rich in vitamin A value, but otherwise unchanged, both males and females increased the concentrations and total amounts of vitamin A in their livers in a fairly regular manner from the age of 30 to that of 300 days.

With the vitamin A value of the diet again doubled both males and females show increased rates and amounts of storage of vitamin A.

It is clear that when the vitamin A value of the food is high enough to furnish a surplus for storage in the liver, the amount stored and the concentration reached, are very significantly influenced both by the level of intake of the vitamin and the length of time that the body has been in daily receipt of a surplus.

The amounts of vitamin A here found agree with those obtained by the single-feeding method as reported in the accompanying paper (Campbell, Udiljak, Yarmolinsky, and Sherman '45) except that at the highest level of vitamin A intake and the two highest ages of the range studied, higher results were found by the method here used. Whether the rats, on this high level of vitamin A intake, changed some of the vitamin A to another form in the liver, which was inactive biologically but determined chemically, is in question.

SUMMARY

The effect of three levels of vitamin A on liver storage of this vitamin in the albino rat at 30, 60, 90, 150, and 300 days of age has been studied.

There was a statistically significant increase in the storage of vitamin A per gram of liver tissue as the level of intake was increased from 3 to 6 I.U. and from 6 to 12 I.U. per gram of diet.

At a level of intake of 3 I.U. per gram, there was very little storage of the vitamin and no increase in storage with increasing age up to 300

days. The vitamin A value of 3 I.U. per gram is not optimal and in the light of these storage results appears to be near the minimal limit of adequacy.

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THE AVAILABILITY OF ASCORBIC ACID IN PAPAYAS AND GUAVAS[1]

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TWO FIGURES

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Papayas and guavas are unusually rich sources of ascorbic acid (Miller et al., '44) surpassing even the citrus fruits. Since large amounts of these fruits are available in the Hawaiian Islands and elsewhere it is possible for them to make a significant contribution toward providing one of the essentials of an adequate diet. There is no information, however, on the availability to human beings of the ascorbic acid of these fruits. The experiments to be described in this paper were carried out in order to provide this information.

The plan of experiment was similar to that used by Clayton and Folsom ('40), Todhunter and Fatzer ('40) and by Clayton and Borden ('43). The data obtained by these workers indicated in general that the ascorbic acid provided by the foods studied was just as available to the body as was ascorbic acid taken in the pure form. The general principle of this method is to compare the excretion of ascorbic acid by subjects on an ascorbic acid-low diet: (a) when they are receiving the test foods; and (b) when they are receiving an equivalent amount of synthetic ascorbic acid.

Various workers refer to the difference between the amount of ascorbic acid ingested and that excreted as the amount utilized, but such a statement assumes that the ascorbic acid is completely available. Because of the possibilities of incomplete absorption or partial destruction in the intestinal tract this assumption is not justified. It has been clearly demonstrated, however, that the amount of ascorbic acid excreted in the urine increases when the amount ingested increases. From metabolism studies such as reported here, therefore, one may conclude that if equal excretions of ascorbic acid result when different sources of ascorbic acid are ingested, then the availability of the ascorbic acid to the body

[1] Published by permission of the Director of the Hawaii Agricultural Experiment Station as Technical Paper no. 125.

must be equal. Furthermore this is true only if all other experimental conditions which might influence the excretion of ascorbic acid, such as the state of saturation of the body tissues, are kept constant. This is the assumption made in the present study in which two series of experiments involving fourteen different subjects were carried out.

METHODS

The diet. A balanced and varied diet [2] providing an average of less than 5.0 mg. of ascorbic acid per day was fed. The intake of foods containing small amounts of ascorbic acid was carefully controlled, but the subjects were allowed as much as they wanted of all other foods. Canned fruits, vegetables and evaporated milk were purchased in case lots and samples were assayed for vitamin C by dye titration. Fruit and vegetable juices were discarded and the vegetables were reheated in relatively large amounts of water which were also discarded. Fruits and vegetables used were canned apples, pears, peaches, figs, sweet corn, green beans, carrots, and beets; dried prunes, raisins, dates and figs; and raw apples without the skins. Since the diet was low in protective foods additional vitamins were provided in the form of capsulated preparations.

The papayas (Carica papaya — solo variety) weighed about one pound each and were grown on the Station farm at Poamoho. The guava juice was prepared according to the method of Miller et al. ('37) from common guavas (Psidium guajava) growing wild on the island of Oahu. The ascorbic acid content of the papayas ranged from 60 to 100 mg. per 100 gm. and that of the guava juice (a watery extract of cooked guavas) from 60 to 90 mg. per 100 ml.

Collection of samples. Twenty-four-hour urine samples were collected in brown glass bottles containing toluene and enough concentrated oxalic acid to bring the acid concentration of the final volume to 0.5%. Creatinine was determined daily on each sample as a check on the accuracy of the collections.

Ascorbic acid determinations. All ascorbic acid determinations were made by dye titration with the exception of plasma samples and foods yielding colored extracts which were assayed with the aid of a photoelectric colorimeter. The test foods were assayed each day. Individual papayas were assayed by taking at least four thin lengthwise slices as a composite sample. The individual portions given to the subjects were also in the form of lengthwise slices.

[2] Detailed records are on file in the office of the Nutrition Department of the Experiment Station and further information concerning the diet will be supplied upon request.

PLAN OF EXPERIMENTS

Experiment I. Nine subjects (4 females and 5 males, 19 to 33 years of age) serving from 3 to 12 weeks each were used in this experiment. Each subject was given 300 mg. of ascorbic acid per day for 3 days before starting the experiment. The experimental periods were as follows:

Saturation period — 1 day. Sunday. Regular diet at home plus 300 mg. ascorbic acid in forenoon.

Control period — 6 days. Monday through Saturday. Basal diet plus 75 mg. synthetic ascorbic acid per day.

Experimental periods — 6 days. Monday through Saturday. Basal diet plus one of the test foods in an amount providing 75 mg. of ascorbic acid per day.

All of the subjects except one repeated the control period at least twice. Two subjects received synthetic ascorbic acid in amounts other than 75 mg. per day for one or more periods in order to estimate the sensitivity of the method. All supplements were taken at 5:00 P. M., just before the evening meal except on Saturdays when they were taken at 12:00 noon.

Experiment II. Six subjects (3 females and 3 males, 29 to 34 years of age), one of whom had also served in experiment I, took part in this experiment. Each subject took 100 mg. of synthetic ascorbic acid per day for 4 days preceding the experiment. The subjects remained on the basal diet plus a constant intake of 75 mg. of ascorbic acid per day without interruption for 5 weeks. The supplements given during the 6 weeks of the experiment were as follows:

Preliminary adjustment period:

Week 1 — 6 days. 75 mg. synthetic ascorbic acid per day.
Week 2 — 7 days. 75 mg. synthetic ascorbic acid per day.

Experimental periods:

Week 3 — 7 days. Papaya equivalent to 75 mg. ascorbic acid per day.
Week 4 — 7 days. 75 mg. synthetic ascorbic acid per day.
Week 5 — 7 days. Guava juice equivalent to 75 mg. ascorbic acid per day.

The subjects in series II received their supplements at 5:00 P. M. each day throughout the experiment. Blood samples were taken on the afternoon of the sixth day of each period.

RESULTS AND DISCUSSION

Experiment I. The daily excretion of ascorbic acid for this group varied greatly due largely to the plan of experiment used. The day-to-day variations in excretion of ascorbic acid are illustrated in figure 1 which presents the individual 24-hour excretion values for subject D. On Mondays (first day of each period) the excretion values were generally quite high due to the high intake of the previous day. They then decreased throughout the week but frequently rose slightly on Saturday due to the change in time of administration of the supplement. The average excretions for each period may be compared, however, since

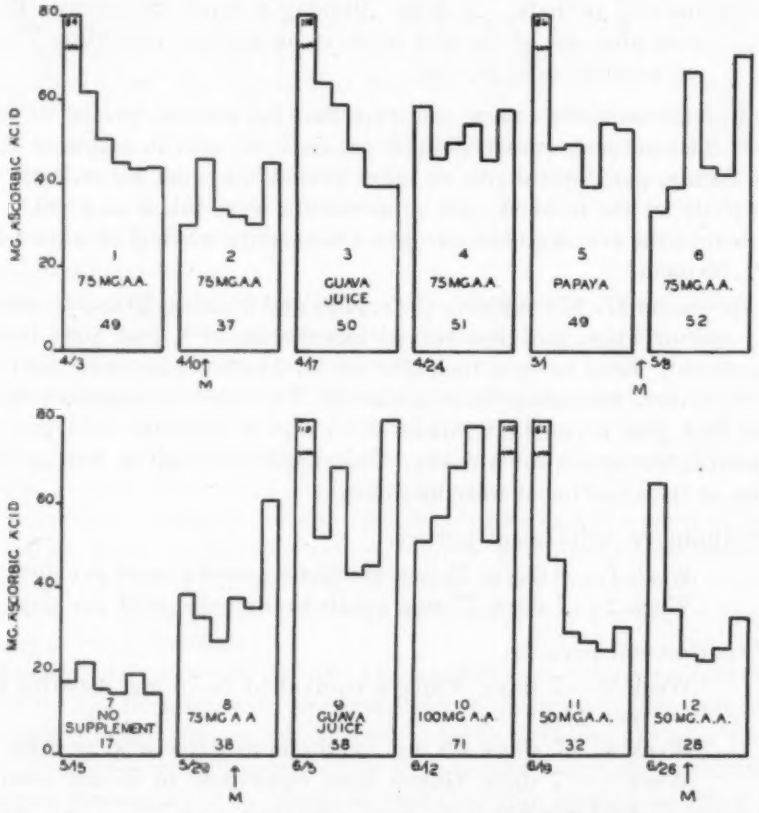

Fig. 1 The excretion of ascorbic acid by subject D during twelve supplemental periods. Each block represents on period of 6 days. The supplements given and the average excretion value for each period are written in the base of each block. Each bar represents the milligrams of ascorbic acid excreted in 24 hours. Broken bars represent excretions greater than 80 mg. The arrow and letter M indicate the first day of menstruation.

each period followed the same plan and each subject acted as his own control.

Table 1 shows the average excretion of ascorbic acid per period for each subject. The excretion values for the first day of each week were so extremely variable that they were omitted from the averages. The values given in the table, therefore, represent the average daily excretion for 5 days only.

Considerable variation occurred within subjects as well as between subjects. Two subjects, F and G, showed very little variation among

TABLE 1

Comparison of the excretion (5-day averages) of ascorbic acid by subjects of experiment I when receiving 75 mg. of ascorbic acid per day in the form of synthetic ascorbic acid, papayas or guava juice.

SUBJECT	AVERAGE EXCRETION PER PERIOD				DIFFERENCE		
	Control periods		Experimental periods		Maximum between control periods	From control periods	
	Individual periods	Average	Papaya	Guava juice		Papaya	Guava juice
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
A	51						
	61	56	65	64	10	+9	+8
B	57	60	59	55	6	−1	−5
	63						
C	43	48	53	41	9	+5	−7
	52						
	49						
D	49	51	49	50	3	−2	−1
	51			58			+7
	52						
E	45	46	53	40	6	+7	−6
	44						
	50						
F	43	44	45	44	1	+1	0
	44						
G	31	31	29	29	..	−2	−2
H	48	49	42	40	2	−7	−9
	50						
J	35	24	32	28	17	+8	+4
	18						
	20						
Av.		45	47	45	5	5	5

their average excretion values, but in the case of certain other subjects, (A, C, H, and J) the differences were large. The reproducibility of the results can be judged by noting the range of values for the control periods of a single subject.

An estimation of the significance of the differences between supplements can be made by comparing the values shown in columns 7 and 8 with those in column 6 of table 1. Column 7 which compares the excretions on papayas with the average excretions on synthetic ascorbic acid contains values ranging from 1 to 9. The values in column 8 which compares guava juice with synthetic ascorbic acid range from 0 to 9. Since these differences were no greater than those between individual control periods (1 to 10) they cannot be considered to be significant. It may be concluded, therefore, that within the limits of error of the method the ascorbic acid of guava juice and papayas was just as available as synthetic ascorbic acid.

In order to estimate the sensitivity of the method, subjects D and G served one or more periods in which they received 50 or 100 mg. of ascorbic acid per day, a variation of $\pm$ 30% from the usual control level. The differences between the excretions at these levels and the excretions at the 75-mg. level ranged from 19 to 38 mg. These differences were much greater than those between periods at the 75-mg. level and indicate that differences of $\pm$ 30% in the availability of ascorbic acid could readily be detected by the method used.

Experiment II. In this experiment the subjects after being maintained on a relatively high intake of ascorbic acid for 4 days were then held on a constant intake of 75 mg. per day (plus the small amount in the basal diet) throughout the 5 weeks of the experiment. This plan was adopted in an effort to reduce the daily fluctuations in the quantity of ascorbic acid excreted and thus to obtain data of greater significance. The individual 24-hour excretion values for all subjects of this experiment are shown in figure 2. Inspection of this figure reveals that all subjects excreted much more ascorbic acid during the first week than in later periods and in most cases a relatively constant level was not reached until after 2 weeks. For this reason the first 2 weeks are considered to be an adjustment period and only the last 3 weeks were used for the purpose of this experiment.

Fig. 2 The daily 24-hour excretions of ascorbic acid by subjects on experiment II. The figures inside the blocks show the average excretion for the period. The numbers 1 to 5 along the ordinate indicate weeks and supplements as follows: 1 and 2 — Adjustment period — 75 mg. synthetic ascorbic acid; 3, 4 and 5 — Experimental period — 3 — Papayas, 4 — 75 mg. synthetic ascorbic acid, 5 — Guava juice. The arrow and letter M indicate the first day of menstruation. D, K, and L — females; M, N, and O — males.

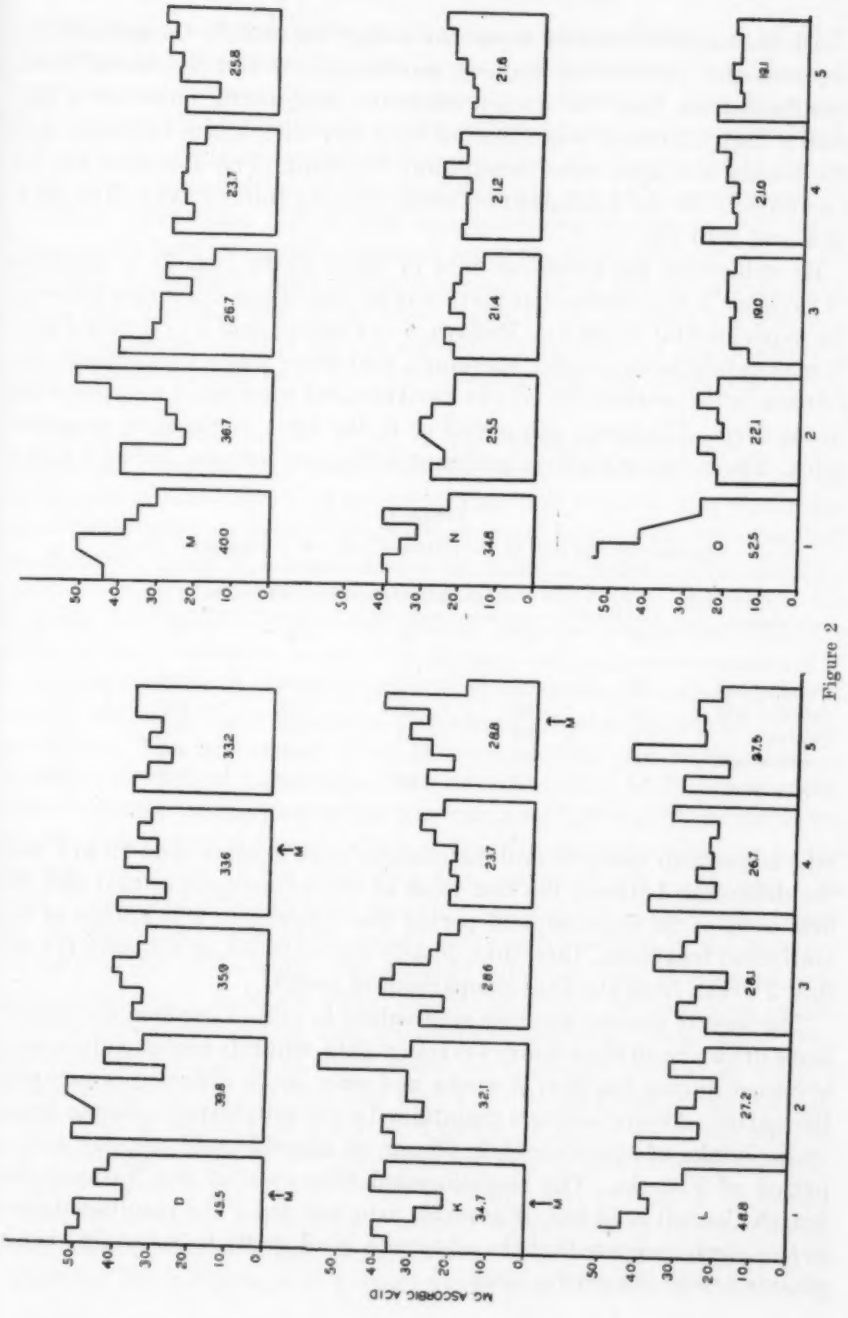
MG ASCORBIC ACID
D
43.5
398
359
336
332
K
34.7
321
286
237
288
L
48.8
27.2
281
267
375
M
400
367
267
237
258
N
348
255
214
212
216
O
525
221
190
210
181

Figure 2

All the subjects showed some day-to-day fluctuation throughout the experimental periods but the men, particularly O and N, showed much less fluctuation than the women subjects. In general, however, a day with a high excretion was followed by a day with a low excretion and the weekly averages were remarkably constant. The averages for all six subjects for the 5 consecutive weeks were as follows: 42.7, 30.6, 26.6, 25.4 and 26.0 mg.

By subjecting the excretion data of the 5 weeks (fig. 2) to analysis of variance it was shown that there was no significant difference between the experimental weeks 3 — Papaya, 4 — Control, and 5 — Guava Juice. It may safely be concluded, therefore, that there was no significant difference in the availability of the ascorbic acid whether it was provided in the form of the pure compound or in the form of papayas or guava juice. There was a highly significant difference between weeks 1 and 2

TABLE 2

The ascorbic acid level of the plasma of subjects in experiment II.

SUPPLEMENT	MILLIGRAM PER CENT ASCORBIC ACID					
	D	K	L	M	N	O
Ascorbic acid	1.3	1.3	1.3	1.0	1.4	1.3
Ascorbic acid	1.2	1.3	1.3	0.7	1.3	1.0
Papaya	0.9	1.1	1.1	0.7	0.9	0.9
Ascorbic acid	1.0	0.9	1.0	0.7	0.9	0.7
Guava juice	1.1	1.1	0.9	0.9	0.9	0.9

(the adjustment period) and the chances were greater than 19 to 1 that the difference between the last week of the adjustment period and the first week of the experimental period was significant. The results of the statistical treatment, therefore, justify the omission of the data for the first 2 weeks from the final comparison of results.

The weekly plasma ascorbic acid values in table 2 confirm the conclusions drawn from the urinary excretion data. Slightly higher values were obtained during the first 2 weeks and only small differences occurred thereafter. All six subjects maintained very satisfactory plasma levels on an intake of approximately 80 mg. of ascorbic acid per day over a period of 5 weeks. The recommended allowance of the National Research Council is 75 mg. of ascorbic acid per day. The results obtained in this study indicate that the allowance is adequate to maintain normal plasma levels of ascorbic acid.

Effect of acidity or alkalinity. Various workers, Hawley et al. ('37), Goddard and Preston ('38) and Meyer and Hathaway ('44), have attempted to determine the effect of acidity or alkalinity upon the excretion of ascorbic acid, but their results so far have not been conclusive. No attempt was made in our experiments to study this factor and it was not rigidly controlled although the basal diet did not vary greatly and the subjects were asked not to take any alkalizing medicines. Any effect which may have occurred was small since the fruit supplements in the amounts used would have changed the acidity of the urine very little. This was demonstrated by an experiment to be reported elsewhere in which the ingestion for 3 days of amounts of papaya and guava juice five to ten times those given in this experiment caused only small changes in the pH of the urine.

Effect of menstruation. Early in the course of experiment I it was noted that very low values were obtained just before and/or during the early part of the menstrual period. From that time on care was taken to have the subjects receive synthetic ascorbic acid during these periods so that they could be compared with the control periods. The excretion values during these periods, in cases where they were well below the average for the individual, were omitted from the comparisons made in table 1.

Figure 1 which shows the daily excretion values of subject D for 12 weeks, including four menstrual periods, illustrates the effect of menstruation. The depressing effect of menstruation was pronounced in the second week as indicated by the average value of 37 mg. as compared with the normal value for this subject of 51 mg. Also the value for the first day of the week, which ordinarily was very high due to the saturation period of the preceding day, was very low — 30 mg. The next menstrual period which occurred during the sixth week began on the day of the saturation period and again resulted in a low value on the first day of the week. The average value for week 6, however, was normal (52 mg.) since the value for Monday was not included in the 5-day average and several high values occurred later in the week. The third menstrual period in week 8 was very similar to that in week 2 with a low value on the first day and a low average of 38 mg. for the week. During weeks 1, 2, 3, 4, 5, 6, 8 and 9, in all of which the subject was receiving 75 mg. of ascorbic acid per day in one form or another, no daily values below 35 mg. occurred except just preceding or during menstrual periods. During these menstrual periods there were seven daily values below 35 mg., two of which occurred on Mondays. The values for the 5 Mondays of normal weeks ranged from 58 to 118 with

an average of 93 mg. of ascorbic acid while those which occurred in the 3 weeks of menstruation ranged from 30 to 38 with an average of 34 mg. ascorbic acid. Because of the magnitude of these differences it is not reasonable to suppose that they occurred by chance.

In experiment II, however, little or no effect of menstruation was noted. No satisfactory explanation is apparent for this discrepancy between the observations made in the two experiments. In experiment II the concentration of ascorbic acid in the tissues was undoubtedly lower than in experiment I because of the lack of weekly 1-day saturation periods, but otherwise the conditions were similar. Subject L in this group was in the first month of pregnancy so menstruation was not a factor, but this subject showed more fluctuation than any other.

No mention of any effect of menstruation on the excretion of ascorbic acid has been made by other workers studying the availability of ascorbic acid. Pillay ('40), however, observed a relation between ovulation time and minimum excretion of ascorbic acid and Mickelsen et al. ('43) noted an increase in the plasma ascorbic acid level of women during the middle of the menstrual cycle. From the data accumulated in this study it would appear that there is some relation between menstruation and ascorbic acid excretion. It may be of little significance from the viewpoint of the nutritionist, but it is a factor to be considered in carrying out ascorbic acid metabolism studies.

SUMMARY AND CONCLUSIONS

The availability of the ascorbic acid of papayas and guava juice was determined by comparing the urinary excretion of ascorbic acid by human subjects maintained on a diet low in ascorbic acid: (a) when receiving 75 mg. of synthetic ascorbic acid per day; and (b), when receiving an equivalent amount of ascorbic acid in the form of papayas or guava juice. Two experiments involving a total of fourteen subjects (eight men and six women) were carried out.

The two experiments differed from each other only in the way in which the experimental periods were divided. In the first experiment 6-day periods preceded by a 1-day saturation period were used. In the second experiment the subjects remained continuously on the basal diet plus 75 mg. of ascorbic acid for 5 weeks, the first 2 weeks serving as a preliminary adjustment period and only the last 3 weeks being used for purposes of comparison. This latter procedure greatly reduced the variation in the data obtained.

No significant differences in the availability of the ascorbic acid of papayas or guava juice as compared with synthetic ascorbic acid were found in either experiment.

Blood plasma ascorbic acid levels which were determined in experiment II were similar in all experimental periods. Subjects receiving 75 to 80 mg. of ascorbic acid per day over a period of 5 weeks maintained plasma levels of from 0.7 to 1.0 mg. %.

In some instances menstruation appeared to have a depressing effect on the excretion of ascorbic acid.

ACKNOWLEDGMENTS

Grateful acknowledgment is made to Prof. Carey D. Miller, Head of the Nutrition Department, who suggested the problem and made many helpful suggestions, to Dr. E. L. Willett of the Division of Animal Husbandry who carried out the analysis of variance, to Miss Winifred Ross, Junior Nutritionist, who planned and supervised the preparation of meals in experiment II and Dr. E. A. Fennel and staff at the Clinic of Physicians and Surgeons who took the blood samples. The author also wishes especially to thank the various members of the Experiment Station Staff who served as subjects for these studies.

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NUTRITIONAL STUDIES WITH THE DUCK

II. PYRIDOXINE DEFICIENCY [1]

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ONE FIGURE

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Pyridoxine deficiency has been studied quite extensively in four species: rats, dogs, pigs, and chicks. The deficiency symptoms apparently common to these species (Lepkovsky, '42) are lack of growth, anemia, and convulsions or fits. Convulsions, however, are not consistently noted in all laboratories (Briggs et al., '42), and the anemia is apparently mild or variable in rats and chicks (Fouts and Lepkovsky, '42; Kornberg, Tabor and Sebrell, '45; Luckey et al., '45). Acrodynia is observed in deficient rats but not in the other species. A disturbance in tryptophane metabolism resulting in the excretion of large amounts of xanthurenic acid was first observed in rats by Lepkovsky (Lepkovsky and Nielson, '42; Lepkovsky, Roboz and Haagen-Smit, '43) and has been noted and studied in swine by Cartwright et al. ('44). Deficient dogs, however, excrete very little xanthurenic acid, and none was noted in the droppings of deficient chicks (Lepkovsky, Roboz and Haagen-Smit, '43). It thus appears that certain symptoms may be considered as characteristic of pyridoxine deficiency while others may at this time be considered as only species specific.

Pyridoxine deficiency is easily produced in young ducklings by the omission of pyridoxine from a purified ration previously described (Hegsted and Stare, '45). In this paper we wish to present the results of studies of this deficiency in this species.

EXPERIMENTAL

The pyridoxine deficient ration which was fed ad libitum had the following percentage composition: sucrose 55.7, casein (SMA vitamin-free) 18, gelatin 10, corn oil 4, salt mixture (Hegsted et al., '41) 5,

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[2] Rockefeller Fellow at the Harvard School of Public Health, 1943 to 1945.

mono-calcium phosphate 1, cod liver oil 2, liver extract [3] 4, and choline chloride 0.3. Crystalline vitamins [4] were added to supply the following amounts per 100 gm. of ration: thiamine 400 μg., riboflavin 800 μg., calcium pantothenate 1.5 mg., biotin 20 μg., and nicotinic acid 3.0 mg. Ten milligrams of alpha tocopherol and 10 μg. of menadione dissolved in corn oil were fed twice weekly by dropper. Control animals received the same diet plus 400 μg of pyridoxine hydrochloride per 100 gm. All of the birds were kept in heated cages with raised screen bottoms as previously described.

Acute deficiency in day-old ducklings

In experiment I the deficient diet was fed to 4-day-old white Pekin ducklings while four others received the same diet to which pyridoxine was added. The animals were weighed daily and observed for symptoms of deficiency. Hemoglobin and red cell counts were made at weekly intervals. Blood smears were also taken and stained supravitally with 0.2% alcoholic Azure II followed by Wright-Giemsa counter stain.

The weight curves and the hematological data are shown in figure 1. After the birds had received the deficient diet for about 3 weeks, one of the birds died. To keep the other birds alive, a small amount of pyridoxine (50 μg. per 100 gm. of ration) was added to the ration for a period of 4 days. The data show that on the ration lacking in pyridoxine the growth was very poor, and this was accompanied by the development of a severe anemia. Data obtained the day before the one duck died showed a hemoglobin value of 3.5% and a red cell count of 900,000 per cu. mm., while figures for the control birds were 7.35% hemoglobin and 1,810,000 red cells per cu. mm.[5] All three of the remaining birds showed a prompt response to this small amount of pyridoxine as shown by the growth curves and by the blood picture. It is apparent that at the time the experiment was ended the birds were again showing anemia.

From the blood smears it was possible to compare the relative size of the cells from the deficient and control birds. The length and width of twenty-five cells in a smear from a deficient bird were measured with a calibrated micrometer eye-piece. They showed a mean maximum length of 11.04 μ, mean maximum width of 6.46 μ. Similar data from a control

[3] Fraction "L" supplied by The Wilson Laboratories, Chicago.

[4] Supplied by Merck and Co., Inc., Rahway, New Jersey.

[5] The following data are the average results obtained with young ducklings 4 to 10 days old which received commercial duck pellets: Hemoglobin — 8.19 gm./100 ml.; red blood cells — 1.98 million per cu. mm.; hematocrit — 28.8 ml./100 ml.; mean cell volume — 141 μ^3; mean cell hemoglobin — 41.8 $\mu\mu$g.; mean cell hemoglobin concentration — 29.4%.

duck were mean maximum length 11.45 μ, and mean maximum width 7.19 μ. Assuming that the cells are perfectly ellipsoidal, the areas were calculated according to the formula: Area $= \pi/4 \times$ length $\times$ width. This gave a mean area of 56.21 $\mu^2 \pm 8.42$ for the cells of the deficient bird compared with 64.67 $\mu^2 \pm 4.12$ for the control. The difference is 8.46 μ^2, a value 4.5 times the standard error of the difference. Thus the

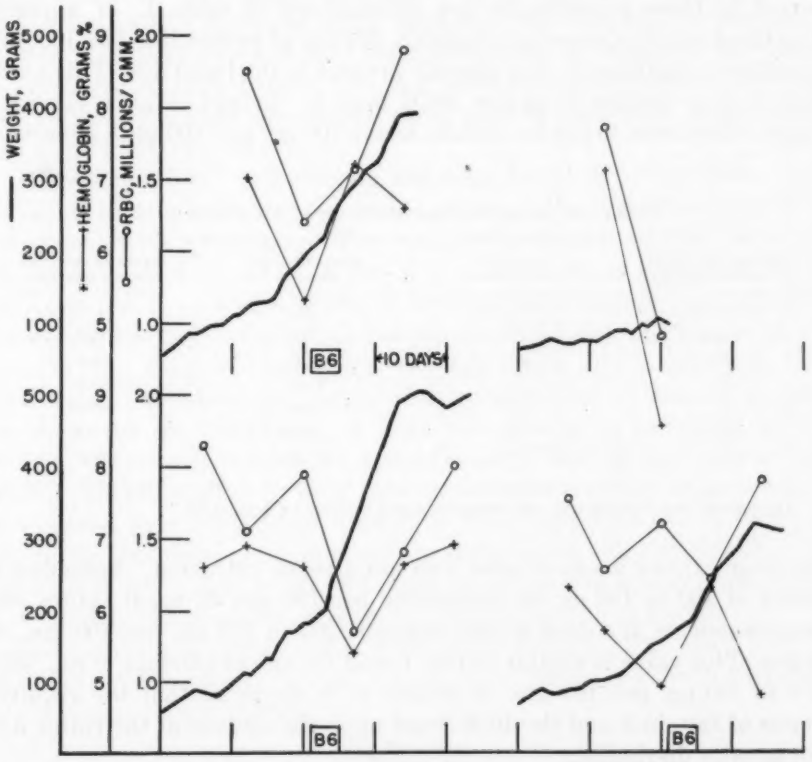

Fig. 1 Data showing the development of pyridoxine deficiency in day-old ducklings. Ration containing 50 μg. of added pyridoxine (per 100 gm.) was fed during the B_6 period.

cells from the deficient birds are significantly smaller than those from the controls. This is also borne out by studies on older ducks reported below.

No clear-cut symptoms other than poor growth and anemia were observed in the deficient birds. They were weak and small, but no dermatitis or nervous symptoms were seen although these were specifically looked for.

Requirements for growth

In an attempt to establish the approximate requirement for pyridoxine, five groups of four ducks each were fed the basal diet to which were added 0, 30, 50, and 100 μg. pyridoxine hydrochloride per 100 gm. of ration, respectively. In a second similar experiment, levels of 50, 75, and 100 μg. per 100 gm. were fed. The results obtained during a 2-week period in these experiments are summarized in table 1. It appears that the duck requires approximately 100 μg. of pyridoxine per 100 gm. of ration in addition to that already present in the basal diet. This basal diet is very similar to ration 483B used by Briggs et al. ('42) with chicks which was found to contain about 100 μg. per 100 gm. However,

TABLE 1

Studies on the pyridoxine requirement of the duckling.

PYRIDOXINE ADDED TO BASAL RATION	NUMBER OF DUCKS	GAIN PER DUCK PER DAY	GAINS AS PER CENT OF CONTROLS
μg./100 gm.		*gm.*	
None	7 [1]	6.9	25.6
30	4	11.6	43.0
50	7 [1]	17.3	64.0
75	4	23.2	86.0
100	7 [1]	26.3	97.5
400	7 [1]	27.0	100.0

[1] Average of two experiments, one group of 4 and another of 3 animals.

the liver extract we have used was not alcohol extracted. Assuming a figure of 100 to 150 μg. of pyridoxine per 100 gm. of basal ration, the requirement of the duck would be about 200 to 250 μg. per 100 gm. of ration. This value is similar to that found for chicks (Briggs et al., '42), 275 to 300 μg. per 100 gm. of ration. It is apparent that the requirements of the chick and the duck based upon the content of the ration are not greatly dissimilar.

Chronic pyridoxine deficiency

Pyridoxine deficiency in four ducks from 2 to 3 weeks old was produced by feeding the same ration. These birds had received duck pellets until they were started on the experimental regime. They showed a failure of growth soon after being placed on the deficient diet. Feathering was poor and practically all of the feathers were lost over the back and sides, and the pinion feathers did not develop. As noted earlier (Hegsted and Stare, '45), poor feather development on this ration may be related

to deficiencies of unidentified factors. A paralysis developed which was characterized by weakness and difficulty in standing. At times the birds sat on their hocks with the head laid on the floor. The toes had a tendency to turn in, and the birds apparently could not extend their toes to stand squarely on their feet. Occasional periods of violent tremors were observed during which the birds shook violently as if suffering from a chill. A few minutes later they might appear quite normal except for a slow uncertain gait or might be quite weak and fall forward on the breast and then be unable to pull their feet forward under the body. The whole picture was variable from day to day, severe symptoms occurring 1 day but relatively normal appearance other than the condition of the feathers a few hours or a day later.

Hemoglobin,[6] red cell counts, and hematocrit determinations were made at intervals during the development of the deficiency and after treatment with pyridoxine. The complete protocols of two birds are shown in table 2. Duck no. 1 was bled at intervals at the beginning in order to put some strain upon the hematopoietic organs and this bird developed the deficiency before the others. The blood picture is characterized by a drop in hemoglobin, red cell count, and hematocrit. The decrease in these three is, however, not proportional so that the anemia, as shown by the calculation of mean cell volume, is generally microcytic. Normal cell volumes of approximately 140 fall to levels as low as 85. There appears to be a slow development of the anemia which is followed by a crisis when the values drop precipitously. In some of the birds, the mean cell hemoglobin and the mean cell hemoglobin concentration fell to low levels. At these times the blood smear was characterized by the presence of large numbers of young red cells.

The response to pyridoxine is exceedingly prompt. The blood picture becomes approximately normal within a few days. A growth response occurs immediately as well.

DISCUSSION

Growth failure is the first symptom of pyridoxine deficiency noted in these animals. The duck is remarkable in the speed with which it develops vitamin deficiencies. Differences in the rate of gain are clearly evident within 3 or 4 days after day-old ducklings are given the deficient ration, in spite of the fact that the basal diet probably contains about

[6] Ten cu. mm. of blood were diluted with 10 ml. of N/10 HCl. After a few minutes standing, 1 ml. of 2N NaOH was added to dissolve the cell stroma after which the hemoglobin content was determined with a Coleman Spectrophotometer at 380 mμ. Readings were compared with a standard curve made from duck blood which had been analyzed for O_2 capacity by the Van Slyke method.

TABLE 2

The blood picture in chronic pyridoxine deficiency and the response to therapy.

	DUCK NUMBER 1								DUCK NUMBER 2							
Date	Body weight	Hemoglobin	Red blood cells	Hematocrit	Mean corpuscular volume	Mean corpuscular Hb	Mean corpuscular Hb concentration	Supplement	Body weight	Hemoglobin	Red blood cells	Hematocrit	Mean corpuscular volume	Mean corpuscular Hb	Mean corpuscular Hb concentration	Supplement
	grams	%	*millions per cubic millimeter*	*ml./ 100 ml.*	*cubic micra*	*micro-micrograms*	%		*grams*	%	*millions per cubic millimeter*	*ml./ 100 ml.*	*cubic micra*	*micro-micrograms*	%	
2/3	490								515							
2/7	550								580							
2/10 [1]	610								695							
2/14 [2]	610	11.0	2.56	37.5	146	41.4	29.4		695	10.1	2.52	34.0	135	40.0	29.8	
2/16 [2]	600	8.8							710	9.6						
2/20	615	6.9							800	8.7						
2/21 [2]		7.2	1.82	27.1	148	39.6	26.2			8.8	2.20	33.5	152	40.0	26.2	
3/1	530	9.8							795	9.4						
3/12	500	5.6	1.73	18.2	105	32.4	31.1		820							
3/13	480	3.5	1.10	10.0	100	31.7	35.0		800	8.2	2.37	32.0	135	34.6	26.6	
3/14								1 mg. B_6 oral								
3/15	540	4.9	1.51	21.0	139	32.5	23.4		814	9.6	2.56	36.5	143	37.4	26.3	
3/30	690	10.4	2.94	34.5	118	35.0	29.8		905	6.8	2.44	27.5	113	28.0	25.0	
3/31	655								880	7.2	2.50	26.5	106	28.8	27.2	
4/2	705	9.5	2.81	32.5	115	33.8	29.2		885	7.6	1.97	26.2	130	37.0	29.0	
4/4	760	8.1	2.42	27.5	114	33.6	29.4		870	6.8	1.78	24.0	135	38.2	28.4	
4/6	695	9.6	2.45	31.0	126	39.2	31.0		890	6.7	1.91	25.2	132	35.0	25.6	
4/9	705	9.7							930	7.1						
4/18	740	8.5	2.09	26.0	124	40.6	32.6		920	4.2	1.86	20.5	110	22.6	20.5	
4/19		8.5								4.4	1.88	21.0	112	23.4	21.0	
4/20	745	8.0	2.34	26.0	111	34.2	30.8		905	4.4						2 mg. B_6
4/21																
4/23	720	7.5	2.58	26.5	99	29.0	28.3		880	4.4	2.00	22.0	110	22.0	20.0	2 mg. B_6
4/24																
4/25										5.1	2.00	24.0	120	25.5	21.3	
4/26	695	8.0	2.34	28.5	121	33.9	28.0		995	7.3	1.90	27.5	145	38.4	26.6	
4/27	655	7.0	2.27	25.0	115	30.8	28.0		990	10.4	2.16	36.0	167	48.1	28.9	
4/30	655	7.1	1.99	25.5	128	35.6	27.9		1010	12.5	2.75	44.0	160	45.5	28.5	
5/4	755	6.4							1260	11.9						
5/6	690	6.0	1.55	20.5	132	38.7	29.3		1250							
5/9	615	4.7	1.42	18.5	130	33.1	25.4		1255							
5/10		4.9		20.5				10 mg. B_6								
5/11	755	6.9	1.63	24.0	147	42.2	28.8		1150							
5/12	855	5.7	1.73	22.0	127	33.0	25.9			10.3						
5/15	980	8.8	2.25	31.0	137	39.1	28.4		1080	9.6						

[1] Started on experimental ration. [2] 15 ml. blood removed.

50% of the total pyridoxine requirement. This is no doubt related to the extremely rapid rate of gain which at some stages is equal to 25% of the body weight per day.

The anemia develops more slowly and paralysis and convulsions appear to be symptoms of chronic deficiency. Periods of convulsion were common in the older ducks which survived long periods upon the deficient ration but were not observed in very young ducklings. Thus some of the difference in species noted earlier may be simply differences in certain of the symptoms related to the age of the animals and relative degree of deficiency. Rations completely lacking in a vitamin may produce acute symptoms much different from those which develop over longer periods of time. This has been clearly shown by Shaw, Phillips and Elvehjem ('45) in vitamin C deficiency in the monkey. It is as yet uncertain whether a derangement of tryptophane metabolism occurs in birds (Lepkovsky, Roboz and Haagen-Smit, '43). Attempts to demonstrate xanthurenic acid in the droppings of severely deficient ducks were unsuccessful.

SUMMARY

Severe acute pyridoxine deficiency in young ducklings is characterized by growth failure accompanied by severe anemia. Neither convulsions nor paralysis were observed in these birds. The pyridoxine requirement of young ducklings is similar to that of chicks, approximately 250 μg. per 100 gm. of ration.

Chronic pyridoxine deficiency in older ducklings produced lack of growth, paralysis, convulsions, severe microcytic anemia, and poor feather development.

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THE EFFECT OF GENETIC VARIATION IN THE FOWL ON THE THIAMINE CONTENT OF THE EGG [1]

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Differences in the vitamin content of eggs and other food products are known to occur as the result of many factors. Of these none are more interesting than those variations which are based on genetic differences. The data presented here show that the thiamine content of the eggs of one of the common breeds of domestic fowl differs markedly from that in two others.

Some years ago, Nichita and Iftimesco ('34), and Nichita, Tuschak and Calcef ('34) found that adult White Leghorns could withstand a deficiency of vitamin B_1 much better than Rhode Island Reds. Since the diets used for the two breeds were not identical, there was some question as to whether these breeds differed genetically in nutritional requirements. Lamoreux and Hutt ('39), using chicks instead of adults, proved that White Leghorns as a breed are more resistant than either Rhode Island Reds or Barred Plymouth Rocks to a deficiency of vitamin B_1. Their experiments gave little clue to the basis for the difference and the present study was made to obtain further information on this point.

PROCEDURE

Whereas in the previous experiments the procedure was to determine how well the test animals could withstand diets deficient in thiamine, in the investigation here reported all birds were given a diet containing ample thiamine and the breeds were then compared with respect to the amount of that substance deposited in their eggs.

While the quantitative requirement of thiamine by mature laying fowls has not yet been determined, it is not likely to be greater than

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that for growing chicks, which Arnold and Elvehjem ('38) found to be 60–80 μg. added per 100 gm. of "low thiamine" feed. Upon analysis, the thiamine content of the grain used in this experiment was found to be 410 μg., and of the mash 540 μg. per 100 gm. of feed. The birds' diet consisted of approximately half grain and half mash, and it is obvious that they consumed an abundance of thiamine.

The eggs analyzed for breed comparisons were laid at Cornell University by pullets varying in age from 7 to 9 months. The flocks of the different breeds were kept under comparable conditions and none of the birds had been laying for more than about 3 months. On December 19, 1944, twenty-four eggs, each laid by a different hen, were taken for analysis. Twelve of these were laid by White Leghorns and twelve by Rhode Island Reds. Two weeks later another twenty-four eggs were taken on 1 day. Of these, half were laid by twelve different Barred Rocks, and the rest by twelve White Leghorns, none of which had contributed to the previous sample. These eggs were all assayed individually, thus yielding determinations for twenty-three different White Leghorn pullets and for eleven of each of the two heavy breeds (one egg was lost through accident in each lot).

Other White Leghorn eggs from a poultry farm near Rochester were analyzed at intervals from January, 1944 to April, 1945. The grain fed this flock contained 400 μg. of thiamine and the mash 580 μg. per 100 gm. of feed. Although analysis was done only on the feed of April, 1945, the hens were presumably on the same diet during the entire period studied. Before analysis the eggs were weighed, broken, and the yolk carefully separated from the white by repeated pourings between the half shells. The white was weighed separately and the total weight obtained after the yolk was added. These egg contents were then mixed in a Waring Blender. Thirty grams of the blended contents were taken as a sample, diluted to 250 ml., acidified to pH 4.0 or below, and stored at about 5°C. All thiamine assays were carried out by the macro-fermentation method of Schultz et al. ('42) as modified by Scrimshaw and Stewart ('44). Nearly half of the samples were routinely checked by two separate assays, including any determinations that seemed to be out of range.

RESULTS

In table 1 the thiamine values are expressed as (1) the amount per egg, (2) the amount per 100 gm. of egg contents (to permit comparison with other determinations thus expressed), and (3) the amount per 100 gm. of yolk. This last figure is probably the best measure of thia-

TABLE 1

Assay of thiamine in eggs of different breeds.

HEN NO.	WEIGHT IN GRAMS			THIAMINE IN MICROGRAMS PER			HEN NO.	WEIGHT IN GRAMS			THIAMINE IN MICROGRAMS PER		
	Whole egg	White	Yolk	Egg	100 gm. egg contents	100 gm. yolk		Whole egg	White	Yolk	Egg	100 gm. egg contents	100 gm. yolk
	I. White Leghorns							II. Rhode Island Reds					
1	55.7	31.0	17.5	50.0	103	286	1	64.4	34.3	22.5	35.8	63	159
2	50.2	29.0	14.8	49.1	111	332	2	50.1	26.5	17.0	31.8	73	187
3	57.0	32.5	17.7	39.7	79	224	3	61.7	37.7	16.5	20.6	38	125
4	52.0	30.3	15.9	54.4	120	342	4	68.1	37.3	23.4	43.1	71	184
5	53.0	31.2	15.6	47.7	102	306	5	62.7	34.2	21.2	38.8	70	183
6	52.1	28.0	18.2	55.4	120	304	6	55.1			35.4	79	
7	51.0	27.9	17.4	50.8	90	234	7	63.6	36.3	20.5	36.4	64	178
8	49.9	26.7	17.6	45.2	102	257	8	62.4	31.8	23.5	38.2	69	163
9	60.1	37.4	15.6	56.2	106	360	9	62.0	36.0	18.0	27.0	50	150
10	55.7	31.6	16.9	39.8	82	236	10	58.1	32.6	18.5	31.2	61	169
11	50.4	29.0	15.2	47.8	108	314	11	61.8	33.0	22.3	38.7	70	174
12	53.7	27.5	19.5	48.9	104	251	Mean	60.9	34.0	20.2	34.3	64.4	167.2
13	48.8	25.8	17.0	36.4	85	214	St. dev.			2.7	6.3	11.5	19.0
14	55.5	29.2	18.3	53.7	133	293		III. Barred Plymouth Rocks					
15	54.6	29.1	19.1	52.5	109	275							
16	55.9	29.2	19.9	51.1	104	257	1	64.3	37.3	20.0	47.0	82	235
17	51.8	27.3	17.8	49.6	110	279	2	59.5	33.0	19.2	34.5	66	180
18	52.7	26.1	20.5	50.8	109	248	3	49.7	27.0	16.6	28.8	66	173
19	49.4	26.3	18.0	47.4	107	263	4	62.7	35.9	18.2	29.8	55	164
20	61.7	34.0	19.2	60.1	113	313	5	60.5	35.4	17.9	28.9	54	161
21	57.0	30.5	17.3	44.5	93	257	6	52.4	28.1	18.3	23.2	50	127
22	53.8	26.8	18.6	52.2	115	281	7	58.7	31.3	19.9	35.8	70	180
23	53.5	26.5	18.7	53.8	119	288	8	60.0	32.7	19.6	39.2	75	200
Mean	53.7	29.3	17.7	49.0	104.5	278.9	9	53.2	28.7	17.9	29.4	63	164
St. dev.			1.5	5.9	11.6	38.1	10	52.8	26.7	19.3	34.5	75	179
							11	58.0	28.0	22.8	36.1	71	158
							Mean	57.4	31.3	19.1	33.4	66.1	174.6
							St. dev.			1.6	6.4	9.9	27.1

mine content for breed comparisons since all of the thiamine is originally deposited in the yolk (Chick and Roscoe, '29; Ellis et al., '33; Baker and Wright, '35; Bethke et al., '36; Scrimshaw and Stewart, '44). If the thiamine content is not described in this manner, consistent differences in the ratio of yolk to total contents may obscure real differences in thiamine content. However, in the fowls studied the percentages of yolk found in the eggs of the different breeds were remarkably similar.

For the White Leghorns the amount of thiamine per 100 gm. of yolk (279 μg.) was 66% greater than that in eggs of Rhode Island Reds (167 μg.) and 60% greater than the figure for Barred Rocks (175 μg.). Application of the t test shows (table 2) that, even with the comparatively small numbers involved, these differences are statistically significant.

TABLE 2

Tests for significance of differences between Rhode Island Reds, Barred Rocks and White Leghorn breed with respect to thiamine content of their eggs.

BREEDS COMPARED	THIAMINE PER EGG			THIAMINE PER 100 GM. EGG YOLK		
	Difference	t	p	Difference	t	p
R.I.R. — B.R.	0.9	.33	.7–.8	7.4	.72	.4–.5
W.L. — R.I.R.	14.7	6.70	$< .01$	111.7	8.73	$< .01$
W.L. — B.R.	15.6	7.07	$< .01$	104.3	8.09	$< .01$
W.L. — both heavy breeds combined	15.2	8.43	$< .01$	107.8	11.18	$< .01$

Whether the thiamine be measured as the amount per egg or as the amount per 100 gm. of yolk, the small differences between the Rhode Island Reds and the Barred Rocks were quite insignificant. In contrast, the values for p of $< .01$ in all the comparisons of White Leghorns with the two heavy breeds show that the excess of thiamine in the Leghorn eggs is highly significant. In the eggs of twenty-three different White Leghorn hens, the thiamine content varied from 214 to 360 μg. per 100 gm. yolk, but in eggs from twenty-two birds of the heavy breeds the range was only from 127 to 200 μg. except for a single egg with an assay of 235 μg.

It should be pointed out that the Leghorns laid somewhat smaller eggs than did the other two breeds, because they had not been laying quite as long as the others. Experience with eggs of these breeds at Cornell has demonstrated that the eggs of White Leghorns laying at maturity are not smaller despite the smaller size of the birds.

The yolk weight was also smaller in the Leghorn eggs than in either of the other two breeds by a statistically significant amount. Since all of the thiamine is contained in the yolk, this should mean less thiamine

in the eggs of White Leghorns. Similarly the heavy breeds would be expected to show more thiamine per egg because of the slightly larger yolks. When the correlation between the thiamine per egg and the weight of the yolk is examined the following coefficients are found: in Leghorns $r = +0.232$, $p = 0.2$–0.3; in Rhode Island Reds and Barred Rocks combined $r = +0.728$, $p = < 0.01$.

While the correlation in the heavy breeds is highly significant statistically, that for the Leghorns is less so. However, both indicate that the amount of thiamine increases with yolk size. The observation that the somewhat smaller yolks of the Leghorns contained more thiamine than the larger yolks of the heavy breeds is all the more striking, because it is in contrast to the relationship shown by these correlations.

TABLE 3

Thiamine content of White Leghorn eggs from a poultry farm.

DATE	EGGS IN SAMPLE NUMBER	MEAN WEIGHT OF CONTENTS IN GRAMS	THIAMINE IN MICROGRAMS	
			per egg	per 100 gm. egg
Jan. 21, 1944	24	54.8	48.2	88
Feb. 24, 1944	36	56.2	51.1	91
Apr. 1, 1944	15	54.6	59.5	109
Oct. 12, 1944	10	50.3	48.3	96
Oct. 27, 1944	10	42.6	42.6	100
Jan. 1, 1945	20	47.6	44.3	93
Jan. 29, 1945	10	51.8	37.3	72
Feb. 8, 1945	10	50.7	40.6	80
Mar. 10, 1945	10	48.5	48.5	100
Apr. 12, 1945	10	50.6	50.6	100
Averages, these Leghorns:		50.8	47	93
Averages, Cornell Leghorns:		47.0	49	105

The question arises whether the comparisons of the breeds might be influenced by the fact that the Barred Rocks and Rhode Island Reds had been laying about 6 weeks longer than the Leghorns when the eggs were taken for analysis. This possibility seems unlikely, because the amount of thiamine in the diet was too far in excess of the bird's requirements to permit any depletion of reserves in the body. Moreover, a series of determinations on White Leghorn eggs from a nearby poultry farm (table 3) showed concentrations of thiamine quite comparable with those for the Cornell Leghorns. None of them approached the low content found in eggs of Barred Rocks and Rhode Island Reds.

These analyses were made at different seasons in the course of 2 years and with the supply flocks in different stages of egg production. Since the seasonal variations are comparatively small, it seems un-

likely that the differences shown in tables 1 and 2 were influenced by anything other than the genetic constitutions of the breeds involved.

DISCUSSION

While the difference between White Leghorns and the other two breeds with respect to the thiamine content of their eggs is quite distinct, the physiological basis for this is not so clear. Ellis and workers ('33) have shown that fowls consuming diets high in vitamin B_1 put from two to three times as much of it in their eggs as do fowls on diets low in that vitamin. Their data suggest that the amount of thiamine found in the egg is some indication of the excess present in the body above the level required for normal physiological processes. If this be so, then among fowls getting the same diet, the ones utilizing the most thiamine for their metabolic processes (in this case, the two heavy breeds) would have the least surplus available for deposition in the egg. Conversely, the comparatively high content of thiamine in eggs of the White Leghorns should indicate a lower requirement for the vitamin in the normal metabolism of this breed.

If the White Leghorns consumed more feed the difference would not be as striking. On the contrary the differences shown in tables 1 and 2 are emphasized by the fact that the total thiamine intake, which is directly related to the amount of feed consumed, was less for the White Leghorns than for the two heavy breeds. The latter were found in extensive comparisons by Waite ('34) to consume about 13 to 15% more feed per year than White Leghorns. These differences would undoubtedly have been still greater if the Reds and Rocks had laid another thirteen to twenty eggs per bird to equal the egg production of the Leghorns. The contrast is also emphasized by the fact that the White Leghorns are known to be more active than hens of the heavy breeds. On this basis they should use more thiamine and have less to deposit in their eggs.

The difference is not due to the greater body mass of the heavy breeds. The average weight of the Leghorns in the flock laying the eggs studied was 1941 gm. in January, 1945. At the same time, the weight of the Rhode Island Reds would be about 2700 gm. and of the Barred Rocks about 2540 gm.[4] The 23–24% greater body mass of the heavy breeds does not account for observed thiamine differences in the egg of 60–66%, particularly when the other factors discussed above are taken into account.

[4] The last two figures are not actual weights but are the averages of fifty or more representatives of each breed weighed several years ago.

Scrimshaw and co-workers ('44) have shown that the thiamine initially deposited in the egg is still present at the time of hatching. The demonstration that White Leghorn eggs contain more thiamine than those of Rhode Island Reds and of Barred Rocks suggests that the greater resistance of Leghorn chicks to a deficiency of thiamine found by Lamoreux and Hutt ('39) might have resulted solely from the greater concentration in their tissue at hatching. This error could not apply to the trials in which all chicks received a normal diet, high in thiamine, before being put on the deficient diets at 2 and 3 weeks of age. In those chicks, as well as in the adult fowls studied by the Roumanian workers, the White Leghorns were consistently more resistant to a deficiency of vitamin B_1 than the other breeds. From these earlier comparisons on deficient diets, together with the present one on diets with ample thiamine, it is clear that White Leghorns must differ from Rhode Island Reds and from Barred Rocks in being able to utilize more efficiently the thiamine in their diets.

While differences between species in nutritional requirements are well known, genetic variability in this respect has been comparatively little studied within species. That strains of the rat differ in requirement of vitamin B_1 was shown by Light and Cracas ('38). In the ascomycete, Neurospora sitophila, most strains can synthesize thiamine, but Beadle and Tatum ('41) found a mutant able to synthesize only the pyrimidine half of the molecule and not the thiazole half. It seems probable that further study will bring to light other cases of genetic variability in the utilization of this vitamin.

In other comparisons with Rhode Island Reds and Barred Rocks, the White Leghorns have proven more resistant to extreme heat (Hutt, '38), more resistant to Salmonella pullorum, (Hutt and Scholes, '41) and superior in regulation of body temperature shortly after hatching (Lamoreux and Hutt, '39). In a discussion of these physiological traits and others (Hutt, '41), it was pointed out that they are inherited characteristics just as much as the more obvious variations in structure and color by which breeds are differentiated. To account for them is difficult, since all are characters for which there could have been no conscious selection by the breeders when the breeds were formed. Since the Leghorns originated in the Mediterranean area, while the ancestors of the Rhode Island Reds and Barred Rocks came from China, the possibility exists that natural selection in different environments was instrumental in bringing about the differences observed.

SUMMARY

Using a modified macro-fermentation assay procedure, the mean thiamine content of the egg was compared in three different breeds of domestic fowl. These were kept under comparable conditions and their diet contained about 5 μg. of thiamine per gram. The thiamine content was found to average 279 (in μg. per 100 gm. of yolk) for White Leghorn eggs, 167 for Rhode Island Reds and 175 for Barred Rocks (105, 64, 66 μg. per 100 gm. of egg contents, respectively).

From these results and other reports for hens on diets deficient in thiamine, it is concluded that the White Leghorns must differ genetically from the other two breeds in the capacity to utilize more efficiently the thiamine in their diet.

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PHYSIOLOGICAL AVAILABILITY TO THE RAT OF CRUDE CAROTENE IN VEGETABLES

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Using the dark adaptation test as a measure of carotene utilization by man Booher and associates ('39 a, b) found that the carotene in spinach was approximately 1.7 times as effective as that contained in peas while the carotene in both of these vegetables was utilized better than when taken in cottonseed oil. Carotene was less efficiently absorbed from carrots than from butter in the human intestinal tract (Kreula and Virtanen, '39).

Lease and co-workers ('39) and Sherman ('40, '41) have observed that the utilization of carotene by the rat in curative growth tests is a function of the solvent. Smith and Otis ('41) reported that the quantity of vitamin A in the livers of depleted rats fed carotene varied with the source. Using essentially the same technic Guggenheim ('44) has demonstrated that the utilization of carotene derived from various plant materials ranged between 33 and 67% of that noted when preformed vitamin A was taken. In the case of lettuce, however, carotene utilization was found to equal that of the preformed vitamin.

Studies during the past several years (Bacharach, '40; Davies and Moore, '41; and Harris and associates, '43) have demonstrated that vitamin E (tocopherol) improves utilization of carotene by protecting it against oxidation primarily in the intestinal tract. In this connection it is of interest that of the vegetables tested lettuce was found to contain the highest amount of vitamin E (Karrer and Keller, '38). Guggenheim ('44) was able to demonstrate that supplementation of the basal carrot diet with tocopherol doubled the biological value of the carotene content. These findings suggest that the variability in responses following the ingestion of carotene derived from different food sources, reported for both the rat and the man, may be due in part to variations in tocopherol intake.

In the present study results are reported on a variety of vegetable products correlating the colorimetrically determined crude carotene

content with biological vitamin A potency. Tocopherol was ruled out as a variable in the biological assays.

EXPERIMENTAL PART

Assay materials. These were canned vegetables prepared from one batch and stored during the period of assay at 5°C. For an evaluation of the physiological availability of carotenes derived from a variety of vegetables it was considered desirable to use canned pureed or chopped vegetables rather than raw products. This avoided uncertainty regarding either the uniformity of the assay doses or the stability of the carotenoids, inasmuch as no active enzymes remained in the heat-processed foods.[1]

Assay procedures. A photometric adaptation of the A.O.A.C. ('40) colorimetric procedure was employed for estimating crude carotene. The standard was β-carotene, recrystallized according to the method of Kemmerer ('41). The test is conducted on the unsaponifiable fraction after the removal of the xanthophyls by extraction of the petroleum ether solution with 90% methanol. When tomatoes were present, the lycopene and xanthophyl in the unsaponifiable extract were first removed by adsorption on specially prepared magnesium carbonate (Fraps and associates, '40). In the International Standard for vitamin A, one unit is 0.6 μg. of pure β-carotene. However, in setting the feeding levels for the bioassays 1 μg. of crude carotene was assumed to have the potency of one unit of vitamin A. Once each week a fresh can was opened for preparation of the assay supplements. The remaining portion was stored at 5°C. under nitrogen. Tocopherol was eliminated as a variable in the bioassays by the daily administration to each rat of 0.1 ml. of cottonseed oil containing 0.3% mixed tocopherols.[2] Since approximately 0.2 mg. tocopherol was supplied daily via the cottonseed oil in the basal diet, the rats received a total of approximately 0.5 mg. tocopherol per day. The results of recent studies (Harris and associates, '43) indicate that the optimal effect of tocopherol supplementation of the basal ration is attained at the level of 0.5 mg. per day.

In order to permit estimates of potency to be calculated from the animal data, the USP reference cod liver oil was fed at three levels, 2, 3 and 4 units per day, and the assay material at an assumed level cor-

[1] Colorimetric carotene analyses (A.O.A.C., '40) of the material removed from the commercial container before and after storage in jars for periods of 2 weeks at 5°C. gave the same values, indicating no loss of the provitamin.

[2] Obtained from Distillation Products, Inc., Rochester, New York. In the fortification of the cottonseed oil correction was made for the purity of the mixed tocopherol solution.

responding to the median dose, 3 units. Each estimate of potency was based on interpolation of the assay response on the curve for the comparable reference groups.

RESULTS

Eighteen samples of ten vegetable products were tested by the colorimetric and biological assay procedures. The results listed in table 1 show good parallelism between the bioassay data and the vitamin A values predicted by the non-biological method. On the average, 1.0 μg. of crude carotene (free from xanthophyl and lycopene) in these vegetables was found to be equivalent to one USP unit of vitamin A.

TABLE 1

Comparable vitamin A activity of carotenes in a variety of heat-processed vegetables.

MATERIAL	SAMPLE NO.	CAROTENE CONTENT [1] (A)	BIOASSAY [2] ESTIMATE OF VITAMIN A POTENCY (B)	RATIO (A/B)
		μg./gm.	*USP units/gm.*	
Carrots	1	62	64	1.0
	2	32	36	0.9
Kale — swiss chard	3	17	17	1.0
	4	20	19	1.1
Mixed greens [3]	5	17	18	0.9
	6	14	18	0.8
Prunes	7	5	6	0.8
Pumpkin	8	25	28	0.9
Spinach	9	29	27	1.1
	10	34	36	0.9
Squash	11	25	17	1.5
Squash — pumpkin	12	19	17	1.1
Tomato [4] product	13	15	14	1.1
Vegetable soups	14	25	32	0.8
	15	20	23	0.9
	16	18	23	0.8
	17	14	16	0.9
	18	13	17	0.8
Mean ratio of carotene content to vitamin A potency				1.0

[1] Crude carotene, free from xanthophyls, estimated colorimetrically (A.O.A.C., '40).

[2] Based on the procedure of the U. S. Pharmacopoeia XII ('42).

[3] Kale, swiss chard and lettuce.

[4] The lycopene and xanthophyl in the unsaponifiable extract were removed by adsorption on a specially prepared magnesium carbonate (Fraps, Kemmerer and Greenberg, '40).

In the case of two fruit products, an apricot-apple mixture and a peach purée, the biological responses were found to be far in excess of those predicated upon the carotene values. The bioassay estimate for the former product was 25 units per gram while the latter was found to contain 12 units per gram. The colorimetric analyses indicated that there were only 4 μg. carotene per gram in each of these products. Since the responses by the animals to the materials fed at the levels suggested by the colorimetric tests were far in excess of that obtained with the maximal dose of the USP reference oil, the assays were repeated at higher assumed levels of vitamin A content. The responses then fell within the reference points obtained with the USP oil so that these bioassay estimates are considered to be trustworthy. The colorimetric values were confirmed by replicate analyses. The results obtained with these two materials indicate that the crude carotene content of fruit products may not be a reliable index of vitamin A potency. Other substances may be present which have vitamin A activity but which are either not initially extracted or are removed by the aqueous or 90% methanol washings of the unsaponifiable extract, or do not absorb light at 450 millimicrons. This problem merits further study.

SUMMARY

In testing heat-processed vegetables good correlation is obtained between the bioassay estimates of vitamin A potency and those predicted from the results of colorimetric analyses for crude carotene, provided that in the rat assay the tocopherol variable is eliminated. In these products 1.0 μg. of crude carotene (free from xanthophyl and lycopene) is equivalent to one USP unit of vitamin A. No great differences are observed in the physiological availability of the carotene derived from these vegetable products. In the case of some fruit products the potency predicted from the crude carotene values may significantly underestimate the biological vitamin A activity.

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MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1946 Award of $1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1946.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1st to December 31st the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the prize be divided between two or more persons. It may also be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1945 must be in the hands of the Secretary by January 10, 1946. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

H. E. Carter
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Urbana, Illinois

Secretary, American Institute of Nutrition

BORDEN AWARD IN NUTRITION

The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1946. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1946. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

W. E. KRAUSS
Ohio Agricultural Experiment Station
Wooster, Ohio

CHAIRMAN, NOMINATING COMMITTEE